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"DEHALOCOCCOIDES ETHENOGENES" STRAIN 195, A NOVEL EUBACTERIUM THAT REDUCTIVELY DECHLORINATES TETRACHLOROETHENE (PCE) TO ETHENE

XAVIER MAYMO-GATELL
CORNELL UNIVERSITY
SCHOOL OF CIVIL AND ENVIRONMENTAL ENGINEERING
HOLLISTER HALL
ITHACA NY 14853-3501

SEPT 1997

FINAL REPORT: AUG 1993 – OCT 1996

AIR FORCE RESEARCH LABORATORY
MATERIALS & MANUFACTURING DIRECTORATE
AIRBASE & ENVIRONMENTAL TECHNOLOGY DIVISION
TYNDALL AFB FL 32403-5323

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TIMOTHY G. WILEY, Maj, USAF, BSC
Biosystems Area Manager

Christine Wagener-Hulme

CHRISTINE WAGENER-HULME, LtCol, USAF, BSC
Chief, Environmental Technology Development Branch

Andrew D. Poulis

ANDREW D. POULIS
Scientific & Technical Info Prog Mgr

Neil J. Lamb

NEIL J. LAMB, Col, USAF, BSC
Chief, Airbase & Environmental Technology Division

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	Sep 1997	Final Report Aug 93 - Oct 96

4. TITLE AND SUBTITLE

"*Dehalococcoides ethenogenes*" Strain 195, A Novel Eubacterium that Reductively Dechlorinates Tetrachloroethene (PCE) to Ethene

5. FUNDING NUMBERS

6. AUTHOR(S)

Xavier Maymo-Gatell

7. PERFORMING ORGANIZATION NAMES(S) AND ADDRESS(ES)

Cornell University
School of Civil and Environmental Engineering
Hollister Hall
Ithaca, NY 14853-3501

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)

AFRL/MLQ
139 Barnes Drive, Suite 2
Tyndall AFB FL 32403-5323

10. SPONSORING/MONITORING AGENCY REPORT NUMBER

AL/EQ-TR-1997-0029

11. SUPPLEMENTARY NOTES

Availability of this report is specified on reverse of front cover. AFRL/MLQ Project Officer: Catherine Vogel (904) 283-6208, DSN 523-6208

12a. DISTRIBUTION/AVAILABILITY STATEMENT

Approved for public release; distribution is unlimited.
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12b. DISTRIBUTION CODE

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13. ABSTRACT (Maximum 200 words)

Tetrachloroethene (PCE) is a human carcinogen, and together with trichloroethene (TCE), is widely used. Due to improper handling, they are among the most frequently found groundwater pollutants. A purified, PCE-dechlorinating enrichment culture was developed. This non-methanogenic, non-acetogenic culture could grow with H2 as the electron donor, indicating that H2/PCE serves as an electron donor/acceptor for energy conservation and growth. A novel anaerobic bacterium which dechlorinates PCE to the non-toxic product ethene (ETH), "*Dehalococcoides ethenogenes*" strain 195, was isolated from this enrichment. This is the first pure culture capable of complete PCE dechlorination. "*D. ethenogenes*" strain 195 is an irregular coccus with an optimal growth temperature of 35°C and pH of 6.8-7.5. Phylogenetic analysis indicates that it is a eubacterium which shows no affiliation to known groups. Electron donors tested other than H2 were not utilized nor were electron donors other than TCE, *cis*-dichloroethene (*cis*-DCE), 1,1-DCE, and dichloroethane, which could be freely interchanged and were dechlorinated to ETH. This organism could not grow on vinyl chloride or *trans*-DCE when provided as sole electron acceptors, but both were dechlorinated cometabolically by cells previously grown on PCE. The reduction of VC to ETH was the rate-limiting reaction to the complete dechlorination of PCE. PCE, TCE, *cis*-DCE, and 1,1-DCE inhibited ETH formation from VC when present, but, at low concentrations, their dechlorination coexisted with ETH production. Cultures grown on *cis*-DCE as sole electron acceptor could not dechlorinate PCE unless PCE and *cis*-DCE were added together.

14. SUBJECT TERMS

Hydrogen donor, anaerobic reductive dechlorination, tetrachloroethene, PCE, anaerobic enrichment culture, methanogenesis, acetogenesis, trichloroethylene, TCE

15. NUMBER OF PAGES

232

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF REPORT	19. SECURITY CLASSIFICATION OF REPORT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	UL

CLASSIFIED BY:

DECLASSIFY ON:

PREFACE

This report was submitted as a dissertation to the Graduate School at Cornell University, Ithaca, NY, in partial fulfillment for the degree of Doctor of Philosophy. The dissertation covers work performed by Mr. Xavier Maymo-Gatell. The effort was partially funded by the Armstrong Laboratory Environmental Quality Directorate (AL/EQ) (now known as the Air Force Research Laboratory, Materials and Manufacturing Directorate, Airbase and Environmental Division, AFRL/MLQ).

This dissertation is being published in its original format because of its interest to the worldwide scientific and engineering community. It covers work performed between August 1993 and January 1998. The AL/EQ Project Manager was Ms. Catherine M. Vogel.

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BIOGRAPHICAL SKETCH

"Only reason can convince us of those three fundamental truths without a recognition of which there can be no effective liberty: that what we believe is not necessarily true; that what we like is not necessarily good; and that all questions are open"
Clive Bell, 1881-1964, Civilization (1928), ch. 5.

Francesc Xavier Maymó i Gatell was born in Barcelona, overlooking the Mediterranean Sea, on the 5th of July of 1966. He attended the Universitat de Barcelona for five enjoyable and required years where, in 1991, he received the degree of Llicenciat en Ciències Biològiques. In the summer of 1989 he came to the USA and worked at Lake Grove School, Long Island, NY. In the summer of 1990 he did an internship in microbiology at the University of Massachusetts at Amherst, under the supervision of Prof. S. Goodwin. From 1990 to 1992 he worked as a research assistant at the Departament de Microbiologia at the Universitat de Barcelona under the direction of Prof. R. Guerrero. In 1991 he was granted a privately endowed fellowship from the "La Caixa" Foundation, to seek an advanced degree in the USA. He came to Cornell University in August 1992 to pursue the accomplishment of a Ph.D. in Microbiology and, in the little spare time left, he and Susanna were married at this university. He finished his Ph. D. in the Spring of 1997 under the supervision of Prof. S. H. Zinder.

To Susanna,

whose intelligence and simplicity have brightened my life forever.

ACKNOWLEDGEMENTS

Many people have influenced both my personality and my work, the latter feeding often from the former. They have all left indelible imprints that contributed to make this Ph. D. possible. I wish I could name them all.

I would like to express my deepest thanks to Susanna, the person with whom I share my life, for her constant support and encouragement, for her patience, love and sacrifices and for helping me accomplish what was once an unattainable dream. I wish to thank my parents; they taught me the discipline and allowed me the freedom to get here. Together with all in my family and friends, they have always supported me in this adventure. I also thank all my friends in the US, specially Ivan Tosques, Jim Alfano and Karin van Dijk, Pav Sethi, Jeff Bonano, Yok Lan Teh, Stefano Ferri, and Indi for making my times in this country so much more rewarding and unforgettable.

I wish to express my sincerest gratitude to Dr. Stephen Zinder, adviser and chairman of my special committee, for giving me the opportunity to come to Cornell University. His trust, excellent academic advice, and support are very much appreciated. I thank Dr. Eugene Madsen and Dr. Jane Gibson, for serving in my special committee. Their constant availability and helpful suggestions have also made this possible. I wish to thank Dr. James Gossett and Dr. Emili Montesinos for their profound insights into my research. I also thank Dr. James Russell, Dr. E. Madsen and everybody in their laboratories for letting me use some of their equipment and for being

so nice to me. I thank my lab partners and all the undergraduate students for contributing, each in their special way, to make my research and my days better. My thanks to Dr. William Ghiorse and Dr. Parthasarathy for sharing their profound knowledge of microscopy. Also my thanks to Barbara Eaglesham, Lynne Anguish, Rhea Garen, Sharon Best, Carole Daugherty and Shannon Caldwell for their technical assistance with microscopy and photomicroscopy. I thank Daniel Miller, Corien Bakermans, Kevin McKenna and Donna Fennell for their studies and technical assistance and also the staff of the Section of Microbiology for their patience and help. This research was supported by the United States Air Force's Armstrong Laboratory, Environmental Quality Directorate, Tyndall Air Force Base, Florida, and by the Cornell Center for Advanced Technology in Biotechnology, which is sponsored by the New York State Science and Technology Foundation, a consortium of industries, and the National Science Foundation.

I shall always be in debt to the "La Caixa" Foundation, Catalonia, for providing the exceptional fellowship that allowed me to come to the U.S. and to Dr. Ricard Guerrero, of the Universitat de Barcelona, for believing in me and for being a truly outstanding and generous mentor.

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LIST OF ABBREVIATIONS

BES ..... 2-Bromoethane sulfonic acid

DBA 1,2-Dibromoethane

DCA 1,2-Dichloroethane

DCE ..... Dichloroethene (the three isomers are abbreviated: *cis*-DCE, *trans*-DCE and 1,1-DCE)

e^- ..... Electron

ETH ..... Ethene

GC ..... Gas chromatogaph

H_2 ..... Hydrogen

HDC ..... Hexadecane

MPN ..... Most probable number

PCE ..... Tetrachloroethene (perchloroethylene)

SS ..... Anaerobic sewage digestor sludge supernatant

TCE ..... Trichloroethene

VC ..... Vinyl chloride

VFA ..... Volatile fatty acids solution

v/v ..... Volume per volume

YE ..... Yeast extract

"Dehalococcoides ethenogenes" strain 195
A NOVEL EUBACTERIUM THAT REDUCTIVELY DECHLORINATES TETRACHLOROETHENE (PCE) TO ETHENE

Xavier Maymó-Gatell, Ph.D.

Cornell University 1997

Tetrachloroethene (perchloroethylene, PCE) is a human carcinogen, and together with trichloroethene (TCE), they are widely used organic solvents. Due to the improper handling of these chloroethenes, they are among the most frequently found pollutants in groundwater.

A purified, PCE-dechlorinating enrichment culture was developed. This non-methanogenic, non-acetogenic culture could grow with H_2 as the electron donor, indicating that H_2/PCE serve as an electron donor/acceptor pair for energy conservation and growth.

A novel anaerobic bacterium which dechlorinates PCE to the non-toxic product ethene (ETH), *"Dehalococcoides ethenogenes"* strain 195, was isolated from this enrichment. This is the first pure culture capable of complete PCE dechlorination. Isolation entailed using the antibiotic ampicillin and supplementing the growth medium with cyanocobalamin, anaerobic digestor sludge supernatant (SS), acetate (as carbon source) and cell-free extracts from mixed cultures. *"D. ethenogenes"* strain 195 is an irregular coccus with an optimal growth temperature of 35°C and pH of 6.8-7.5. Its cell envelope did not react with a peptidoglycan-specific lectin and it contains a protein S-layer.

Phylogenetic analysis of its 16S rDNA sequence indicates that it is a eubacterium which shows no affiliation to known groups.

Electron donors tested other than H_2 were not utilized nor were electron acceptors other than TCE, *cis*-dichloroethene (*cis*-DCE), 1,1-DCE and dichloroethane (DCA), which could be freely interchanged and were dechlorinated to ETH. This organism could not grow on vinyl chloride (VC) or *trans*-DCE when provided as sole electron acceptors, but both were dechlorinated cometabolically by cells previously grown on PCE. The reduction of VC to ETH was the rate-limiting reaction to the complete dechlorination of PCE. PCE, TCE, *cis*-DCE and 1,1-DCE inhibited ETH formation from VC when present, but, at low concentrations, their dechlorination coexisted with ETH production. Cultures grown on *cis*-DCE as sole electron acceptor could not dechlorinate PCE unless PCE and *cis*-DCE were added together.

These findings significantly increase our knowledge of the diversity of organisms capable of reductive dechlorination, knowledge crucial to understanding and manipulating the *in situ* dechlorination of chloroethenes.

CHAPTER ONE

INTRODUCTION

"You people never cared for land or deer or bear. When we dig roots, we make little holes. When we burn grass for grasshoppers, we don't ruin things. We shake down acorns and pinenuts. We don't chop down the trees. But you people plow up the ground, pull down the trees, kill everything. The tree says, "Don't hurt me. I am sore". But you chop it down and cut it up. The rock says, "Don't hurt me. I am sore". But you blast rocks and scatter them on the ground. You people pay no attention. How can the spirit of the Earth like you?... Everywhere you people have touched it, it is sore."

Holy Wintu Indian woman, California, speaking of the destruction of the land by gold and hydraulic mining. (Edited excerpt).

For centuries, the awareness of a polluted environment has been present in the minds of people having to live with the consequences of their industrialized society. From the mining exploitation of metals by Romans to the "Chemical Era", deleterious side effects of important processes have occurred. The Industrial Revolution started in England during the last century resulted in an incredibly rapid improvement in living standards. This improvement was directly bound to industries and discoveries of new, useful materials that were easier and cheaper to make and gave better results than the ones used previously. We ate and dressed better, and traveled faster, but in doing so we didn't take into consideration the dangers coming from the toxic derivatives originating from the production of our commodities, until they started to affect us negatively. The realization that something had to be done came at a

time when these compounds had already deeply affected a large portion of the other life forms on this planet. It was realized in the 1960s and 70s in the United States, and later in other industrialized countries, that recalcitrant xenobiotics had become widely distributed throughout the environment. The widespread pollution of soils, sediments and groundwater created hazardous conditions that still, in great measure, have to be dealt with.

1.1 Chlorinated ethenes: uses, problems and solutions.

Since the 1950s, the production of halogenated compounds in the modern world has grown to quantities so important that can only be understood if we take into account the success with which these substances have been used in our industrialized society. In 1985, over 25 billion pounds of halogenated solvents were produced only in the United States, about five-fold more than what was produced in 1960 (58). Three of the most quantitatively important halogenated compounds produced are chlorinated ethenes: tetrachloroethene (perchloroethylene or PCE), trichloroethene (TCE) and vinyl chloride (chloroethene or VC). PCE and TCE are common organic solvents and chemical feed stocks with attractive properties: they are nearly inflammable and non corrosive, and the fact that they do not pose an acute toxicological hazard has mistakenly induced people to use them quite indiscriminately. These compounds have been utilized in a variety of activities: from commercial and industrial dry-cleaning to metal degreasing of industrial and military machinery and equipment, as well as in the production of brake and carburetor cleaners, electri-

cal insulators and spot removers (9, 11).

In 1985, about 700 million pounds of PCE were produced in the United States (58). It is estimated that about 7.8 billion kilograms of PCE have been synthesized and distributed in the United States since 1945 (1). It is no wonder that with such widespread and intensive use of PCE and TCE, together with their improper handling, storage, and disposal, these volatile organic compounds are among the most frequently found pollutants in groundwater (63).

PCE and VC are human carcinogens (23), while TCE and the rest of chlorinated ethenes: *cis*-dichloroethene (*cis*-DCE), *trans*-DCE and 1,1-DCE are suspected carcinogens. All these compounds are listed as volatile organic contaminants under the 1986 Safe Drinking Water Act Amendments, and they are subject to rigorous regulation because of their long-term toxicological threads (24).

Considerable effort and resources have been expended in trying to remediate large quantities of these chlorinated ethenes in the environment. In the last ten years, important advances have been made in the development of techniques for the clean-up of polluted environments. Environmental engineers have developed both physical and chemical methods to remove chloroethenes from soils and waters (e.g., groundwater extraction followed by adsorption to activated carbon or air-stripping of volatile compounds). Soil is presently remediated by incineration or extraction with solvents. However, these techniques are expensive, can be inefficient, and have different drawbacks. Soil incineration deeply perturbs the environment it intends to remediate, and adsorption and stripping techniques merely

carry the contaminant/s from one place onto another.

By contrast, the degradation of chlorinated ethenes by bacteria (bioremediation/biodegradation) has opened a new field of opportunity for a potentially cheaper, effective and environmentally respectful manner of dealing with this problem. It is true that halogenated compounds are considered xenobiotics (technologically created compounds). Nevertheless, there are more than 200 different halogenated organic compounds which are produced by bacteria, algae and sponges (10, 42, 64), and these substances appear to be degraded by some microorganisms. This indirectly implies that many of the xenobiotics released into the environment during this century may not be as foreign to Nature as we thought they were. It is therefore probable that many different microorganisms may posses the ability to degrade one or more of these xenobiotics (and this is indeed what microbiologists are starting to realize). However present, our ability to biodegrade chlorinated ethenes and other contaminants *in situ* or in special facilities directly depends on the information we are able to determine about the bacteria involved, their nutrient requirements, the optimal biodegradation conditions, the physiology of the processes, and the possible formation of toxic intermediates, all of which need to be better understood. I hope that the work presented in this dissertation will add a few important bricks to the wall of information required to effectively bioremediate chlorinated ethenes.

1.2 Aerobic degradation of chlorinated ethenes.

Even though since the early 80s both microbiologists and envi-

ronmental engineers have pursued the finding of a microorganism able to dechlorinate PCE in the presence of oxygen, there has been no success. Under aerobic conditions, therefore, PCE is considered to be non biodegradable. On the other hand, in many instances TCE, the DCE isomers and VC have been proven to be biodegraded under aerobic conditions by several bacterial species, including pseudomonads and other diverse groups (6, 12, 26, 39, 44, 45, 59).

Organisms capable of degrading ethenes with three or fewer chlorine atoms have been found to contain oxygenases (mono and dioxygenases). These are enzymes often of low specificity which produce unstable intermediates. In the case of chloroethenes, these intermediates are rapidly converted mainly to CO_2 and Cl^- (45).

TCE degrading monooxygenases have been studied in methanotrophs (methane-utilizing, aerobic bacteria). In the laboratory, dechlorination of TCE and its derivatives has been demonstrated in several publications (26, 37, 65). Although not all monooxygenases catalyze TCE degradation (61), several pure cultures of bacteria containing both the soluble and the particulate form of methane monooxygenase have been reported to do so (37, 45, 57).

Dioxygenases have also been demonstrated to be responsible for TCE degradation. These enzymes, present in some heterotrophic bacteria, normally require the presence of an aromatic compound, such as toluene or phenol (43), to be active. TCE degradation mediated by dioxygenases has been reported in enrichment (25) and pure cultures (27, 36, 62), and also in *Escherichia coli*, in which TCE is degraded by a constitutively expressed toluene dioxygenase (67).

Even though it is clear that TCE, the DCE isomers and VC dechlorination occurs under aerobic conditions, the microorganisms responsible for these biotransformations are (with the exception of VC) unable to utilize these chlorinated ethenes as growth substrates. None of these compounds can be used as a source of carbon and/or energy and, therefore, their oxidation is considered to be a fortuitous (cometabolic) process which renders slow rates of degradation. Even so, these cometabolic pathways have been used as a bioremediation technology in above-ground treatment systems (2, 19, 40, 55) and to demonstrate and apply the degradation of TCE *in situ* (34, 51, 52). However, all these attempts have to deal with the difficulty of supplying the indigenous bacteria with both oxygen and methane, as well as with toluene or phenol. An exception to cometabolic degradation is *Mycobacterium* L1 [(32)], which is capable of growing on VC as its only source of carbon and energy. Mineralization of VC has also been demonstrated in groundwater samples (12).

1.3 Anaerobic degradation of chlorinated ethenes.

As described in the previous point, PCE is non-biodegradable under aerobic conditions, and TCE, even though it is cometabolically transformed, is quite recalcitrant. In the absence of oxygen, however, both PCE and TCE are degraded by natural microbial communities, mixed microbial enrichment cultures and pure cultures.

In general, there are four different reaction types that transform halogenated compounds (59). The first two, substitution and dehydrodehalogenation, do not change the oxidation state of the

substrate and, hence, do not require e^- donors or acceptors. In a substitution, the halogen is replaced by a nucleophile, like OH^- or HS^-. In a dehydrodehalogenation, a double bond is formed when a halogen and its concomitant or subsequent hydrogen are removed. The other two reaction types, oxidation and reduction, do require the presence of external e^- donors or acceptors.

Reductive transformations of halogenated compounds are, again, classified in 4 basic reaction types (59): (a) dihalo-elimination, in which two halogens from an aliphatic compound are excluded from the molecule previous to the formation of a carbon-carbon double bond; (b) coupling, in which free radicals are involved; (c) hydrolytic reduction, in which a two-e^- reduction of a polyhalogenated carbon is followed by hydrolysis; and (d) hydrogenolysis, a reductive dehalogenation in which a halogen substituent is replaced by a hydrogen (i.e., $RCl+2H^++2e^-=RH+H^++Cl^-$). The latter is the most common of the four processes encountered and it is the reaction type to which the research presented in this dissertation refers.

Certain anaerobes, including methanogens and sulfidogens, are rich in reduced transition-metal coenzymes (such as hemes, vitamin B_{12}, and the methanogenic cofactor F_{430}) and can carry out a slow cometabolic reductive dehalogenation of chloroethenes and other haloorganics with first-order kinetics (18, 29). Nevertheless, some anaerobes can reduce the carbon-chlorine bond, by hydrogenolysis, as an e^- accepting reaction for energy conservation and growth, essentially a form of anaerobic respiration (41). The $E^{\circ\prime}$ half-reaction values for reductive dechlorination of chloroethenes are +0.36 to

+0.58 v (59), making their reduction more energetically favorable than methanogenesis ($E^{\circ\prime}$=–0.24 v) or sulfate reduction ($E^{\circ\prime}$=–0.22 v). The step-by-step sequence for the reductive dechlorination of PCE to ETH by hydrogenolysis is shown in Fig. 1.1.

Biotransformation of chlorinated and other halogenated aliphatics has been demonstrated under different e^- acceptor regimens: from nitrate-reducing conditions (6), to sulfate-reducing conditions (3), to methanogenic conditions (28), the latter being the most common in which dehalogenation occurs. Since the early studies by Bouwer *et al.* (7), considerable evidence has accrued in studies of anaerobic microcosms and mixed cultures for reductive dechlorination of PCE to TCE (22), DCE isomers (3, 31, 50), or VC (28, 60). More importantly, complete dechlorination to ETH (16, 17, 28), ethane (13, 54), or CO_2 (60) has been reported. Partial and complete PCE dechlorination has been demonstrated also *in situ* (5, 8, 38, 47), although the environmental parameters governing this processes are still poorly understood.

Of the three possible DCE isomers formed from TCE dechlorination, *cis*-DCE seems to predominate over *trans*-DCE, 1,1-DCE being the least significant (4, 46, 47). Also, contrary to what happens under aerobic conditions, in the absence of oxygen higher chlorinated compounds are easier to degrade than lesser chlorinated ones (22). Because the less chlorinated ethenes (mainly VC) are regulated as harmful contaminants, incomplete PCE dechlorination is of little benefit to an anaerobic environment. The clear formation of nontoxic products from PCE, like ETH and ethane, indicate the

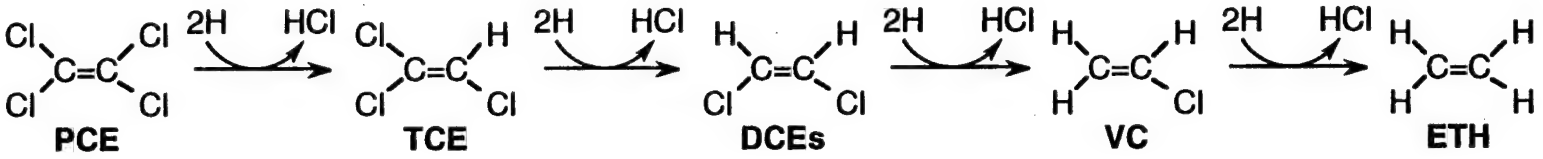

FIGURE 1.1 Sequential reductive dechlorination of chloroethenes. Legend: PCE, tetrachloroethene; TCE, trichloroethene; DCEs, dichloroethene isomers (represented by *cis*-DCE); VC, vinyl chloride; ETH, ethene; 2H, electron pair derived from the e^- donor.

potential for complete anaerobic *in situ* detoxification of chloroethenes, either under existing environmental conditions (natural attenuation), or by addition of e^- donors and nutrients (accelerated bioremediation). However, there needs to be a greater understanding of the organisms and factors involved in the reductive dechlorination process to understand why, for example, some anaerobic systems only partially dechlorinate PCE, while others effect complete dechlorination.

1.4 Reductive dechlorination of PCE by pure cultures.

The microorganisms responsible for reductive dechlorination of PCE and the other chloroethenes are beginning to be identified. Some of these organisms cometabolize PCE to lesser chlorinated products. Probably the best example is *Desulfomonile tiedjei* (15), a sulfate-reducing eubacterium which, coupling growth on formate to the reductive dechlorination of 3-chlorobenzoate, cometabolizes PCE to TCE (20). In studies by Fathepure *et al.* (20, 21) PCE was also cometabolized to TCE by *Methanosarcina sp.* and *Methanosarcina mazei*, both coupling methane production to PCE reduction. The drawback for these cultures is that the rates of chloroethene degradation, as in nearly all cometabolic reactions, are exceedingly slow. For example *D. tiedjei* dechlorinates PCE to TCE at a rate of 2.34 nmol per mg protein per day; *Methanosarcina sp.* and *M. mazei* produced 0.84 and 0.48 nmol TCE per mg protein per day respectively.

Very recently, several organisms have been isolated under anaerobic conditions which can use PCE as a respiratory e^- acceptor,

coupling its reduction to growth. In 1993, Holliger *et al.* described *Dehalobacter restrictus* (formerly strain PER-K23) (33), the main component of a highly purified enrichment culture. This Gram-positive bacterium dechlorinated PCE to *cis*-DCE and it was unable to use any e^- donor provided other than H_2 and formate and only used PCE or TCE as e^- acceptors. This organism dechlorinated PCE optimally at pH 6.8 to 7.6 and at temperatures of 25 to 35°C.

Dehalospirillum multivorans' isolation was described by Scholz-Muramatsu *et al.* in 1995 (49). This organism was isolated from activated sludge using pyruvate and PCE as energy substrates. Its 16S rDNA sequence places it as a new genus into the epsilon subdivision of Proteobacteria (it clusters with Gram-negative sulfate-reducing bacteria and campylobacters). *D. multivorans* grew on several e^- acceptors (fumarate, nitrate and PCE), several other e^- donors (pyruvate, lactate, ethanol, H_2, formate, and glycerol), and acetate as a carbon source. Its growth yield was of 1.6 g cell dry weight per mol chloride released, and it dechlorinated PCE to *cis*-DCE at a rate of 3 µmol per hour per mg cell protein. This organism dechlorinated PCE at an optimal pH of around 7.5 and at a temperature of 25 to 35°C.

Desulfitobacterium sp. strain PCE1 was isolated from an enrichment culture by Gerritse *et al.* in 1996 (30). Its 16S rDNA analysis clustered it with Gram-positive sulfate-reducing bacteria, in the genus *Desulfitobacterium*. Its optimum growth occurred at pH 7.2 to 7.8 and at temperatures of 34 to 38°C. This organism also had a wider metabolic versatility than *D. restrictus*. It utilized as e^- donors: lactate, pyruvate, formate, butyrate, succinate and ethanol; and as e^-

acceptors: PCE, 3-chloro-4-hydroxy-phenylacetate, 2-chlorophenol, 2,4,6-trichlorophenol, thiosulfate, sulfite and fumarate. Strain PCE1 could also grow fermentatively with pyruvate. The main product of PCE degradation was TCE, although small amounts of *cis*-DCE and *trans*-DCE were produced (<5%).

Enterobacter sp. strain MS-1 was isolated from a contaminated site in 1996 by Sharma and McCarty (53). This organism is a facultatively aerobic bacterium that reductively dechlorinates PCE to *cis*-DCE in the absence of oxygen, at a rate of 0.5 μmol per hour per mg (dry weight) of cells. PCE dechlorination requires the presence of glucose, pyruvate, lactate, formate, acetate, yeast extract or a mixture of amino acids as e^- donors. Another facultative aerobe, *Enterobacter agglomerans* biogroup 5, was also reported to reduce PCE to *cis*-DCE (53). Deckard *et al.* (14) reported a different facultative aerobic organism that degrades PCE. Chloride was produced but no intermediates were found and it was suggested that PCE was the source of carbon whereas oxygen served as the e^- acceptor (however, this has not been confirmed).

Strain TT4B, a Gram-negative rod, was isolated from anaerobic contaminated sediments by Krumholz *et al.* in 1996 (35). This strain used acetate or pyruvate as e^- donors and PCE, TCE, ferric nitrilotriacetate or fumarate as the e^- acceptors. Strain TT4B can tolerate levels of PCE of up to 0.1 mM, much lower than the amount tolerated by *Enterobacter* sp. strain MS-1, which was 10 mM.

The metabolic and evolutionary differences existing amongst all these organisms provide an initial view of what is likely to be a high

diversity of organisms capable of reductive dechlorination of chloroethenes. The rates of chloroethene degradation in all these cases are far more rapid than those of cometabolic reactions, which in turn creates authentic possibilities for the anaerobic bioremediation of contaminated sites. None of the microorganisms isolated so far, however, is capable of dechlorinating PCE to a lesser chlorinated ethene than *cis*-1,2-DCE.

1.5 Development of the PCE-to-ETH culture at Cornell University.

In 1989, Freedman and Gossett, at Cornell University's Environmental Engineering Laboratory, were the first to report PCE dechlorination to ETH. With their experiments, they demonstrated the potential for anaerobic dechlorination of low levels of PCE (3 to 4.5 μmol/l) to an environmentally acceptable product (ETH) (28).

The culture was originally obtained from the Ithaca Wastewater Treatment Plant, which has since been replaced by a newer plant. First-generation bottles consisted of 100% material developed from anaerobic digestor sludge and were incubated at 35°C. Subsequent transfers were done under methanogenic conditions with 2 to 10% v/v inocula. Dechlorination of PCE to ETH was observed in all transfers, including the first-generation bottles. An experiment performed on sixth-generation cultures showed that [^{14}C]PCE was being transformed almost exclusively to [^{14}C]VC and [^{14}C]ETH. The last step of dechlorination (VC to ETH) appeared to be rate limiting and, indeed, there was persistence of some VC even after 270 days after the

start of ETH formation by the culture. An e^- donor was required to support dechlorination. Glucose, acetate, formate, and H_2 could be utilized as e^- donors, yet it was methanol that facilitated the greatest conversion of PCE to ETH. 2-bromoethanesulfonate (BES), a selective inhibitor of methyl-coenzyme M reductase (the enzyme which catalyzes the last step of methanogenesis), stopped the formation of methane and, at the same time, the dechlorination of PCE further than TCE. This led to the hypothesis that a methanogen was responsible for the dechlorination of PCE.

As the PCE doses administered to the methanol/PCE culture gradually increased, this enrichment culture evolved to a point where nominal concentrations of up to 550 µmol/l PCE were being almost completely dechlorinated (99% ETH, 1% VC) in just 4 days (17). In these experiments by DiStefano, Gossett and Zinder, VC was still accumulated by the culture and its reduction proved to be the rate limiting reaction.

The high amounts of PCE added to the methanol/PCE culture caused a decline in methane production (at a PCE concentration of about 250 µmol/l) and nearly completely inhibited methanogenesis (at a PCE concentration of about 550 µmol/l). This was supported by unpublished results by Zinder (66) in which populations of methanol-using methanogens were undetectable when the level of PCE was increased to 550 µmol/l, while they were present at populations near 10^7 cells/ml when the PCE level was low. Inhibition could have also been caused by ETH, as it had been demonstrated previously by Schink *et al.* (48). Even though the high PCE/ETH concentrations did

inhibit methanogenic activity, they did not affect dechlorination and the culture could be transferred indefinitely under these conditions. This result strongly pointed towards an identity for the dechlorinating organism/s different from that of methanogenic bacteria, therefore invalidating the initial hypothesis.

The levels of methanol amended as an e^- donor to the culture were low relative to the amount of PCE when compared with the experiments by Freedman and Gossett (28). They represented about twice the amount needed to effect complete dechlorination of the PCE added to ETH (2:1 methanol:PCE ratio, on an e^- equivalent basis). Because methanol is a direct precursor of methane, the absence of methanogenesis produced a more efficient use of the methanol equivalents by both the reductive dechlorination and the acetogenic processes, the activities of which increased considerably after methanogenesis ceased. Still, though, the dominant pathway occurring in the methanol/PCE enrichment culture was acetogenesis (in an e^- balance, acetate production accounted for 69% of the methanol supplied). The microorganism(s) responsible for the reduction of chloroethenes in the culture could be an acetogen, using methanol as e^- donor, or an independent, different organism, which could be using the hydrogen generated during acetogenesis (17).

In further studies, methanol-fed and H_2-fed cultures were tested for PCE dechlorination in the presence of vancomycin, a eubacterial cell wall synthesis inhibitor (16). Vancomycin (100 mg/l) inhibited PCE dechlorination from methanol and acetogenesis from methanol and H_2, but PCE dechlorination from H_2 was not affected. This

suggested that the PCE dechlorinator was resistant to vancomycin and could not use methanol directly, but rather, required methanol metabolism, most likely from acetogenic bacteria to provide H_2 (or formate) as the true e^- donor for reductive dechlorination. In these same studies, addition of BES to the culture again inhibited dechlorination beyond TCE in both methanol- and H_2-fed systems. Since BES, apart from being structurally similar to coenzyme M, is also a halogenated aliphatic like the chloroethenes, inhibition of dechlorination appeared to be due to this resemblance.

DiStefano *et al.* found that for the methanol/PCE culture, H_2 could readily replace methanol for several feedings (16), although eventually performance faltered. Good performance was restored by resuspension of the H_2/PCE culture in the filter-sterilized supernatant from the methanol/PCE culture, suggesting that the latter contained growth factors required by the dechlorinator(s).

More recent time-course studies (56) showed that the methanol/PCE culture was capable of reductive dechlorination of all chloroethenes. PCE, TCE, *cis*-DCE, and 1,1-DCE showed essentially near zero-order kinetics. PCE was initially degraded quantitatively to VC. VC dechlorination to ETH did not usually commence until other chloroethenes (except *trans*-DCE) were absent, suggesting chloroethene inhibition of VC dechlorination to ETH. Dechlorination of VC and *trans*-DCE showed first-order kinetics. The culture also made small amounts of methane after PCE depletion, apparently due to lack of inhibition of methanogenic bacteria at low PCE concentrations. The culture also rapidly dehalogenated 1,2-dibromo-

ethane (DBA) and 1,2-dichloroethane (DCA) to ETH.

1.6 Objectives of this research.

The studies reported so far with the "Cornell Culture" have been done with mixed enrichment cultures in which the specific organisms and interactions that reductively dehalogenate chlorinated ethenes have not been elucidated. The knowledge crucial to understanding and manipulating the *in situ* dechlorination of chloroethenes needs to be complemented by the study of pure cultures; their growth requirements, their diversity, their degradation pathways.

This study had three main objectives:

(a) The isolation of the microorganism(s) responsible for the complete reductive dechlorination of PCE in the methanol/PCE culture. Because the organisms were growing in a poorly defined medium, an initial characterization of the organisms and specific conditions that favor the dechlorination process were performed in the mixed culture. This helped in a direct way in the isolation process.

(b) The characterization of the isolate(s)s structural properties (by optical and electron microscopy), growth requirements (growth rates, e^- donors and acceptors, carbon sources, optimal pH and temperature, etc.), and phylogeny (by analysis of its 16S rDNA).

(c) The quantitative study of the utilization of PCE and its derived molecules as potential e^- acceptors by the isolate.

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CHAPTER TWO

CHARACTERIZATION OF AN H_2-UTILIZING ENRICHMENT CULTURE THAT REDUCTIVELY DECHLORINATES PCE TO ETH IN THE ABSENCE OF METHANOGENESIS AND ACETOGENESIS

"When one is drunk with a vision, he deems his faint expression of it the very wine"

Kahlil Gibran, Sand and Foam, 1970, p. 13
Alfred·A·Knoft, ed., New York

2.1 ABSTRACT.

Previous results indicated that H_2 was the direct electron donor for reductive dechlorination of PCE to ETH by an anaerobic enrichment culture which used methanol as a general e^- donor. Most probable number counts performed on this culture indicated high numbers ($\geq 10^6$/ml) of sulfidogens, methanol-utilizing acetogens, fermentative heterotrophs, and PCE dechlorinators using H_2. A H_2/PCE enrichment culture was derived from a 10^{-6} dilution of the methanol/PCE culture. This H_2/PCE culture used PCE at increasing rates over time and could be transferred indefinitely with H_2 as the e^- donor for PCE dechlorination, indicating that H_2/PCE can serve as an e^- donor/acceptor pair for energy conservation and growth. Sustained PCE dechlorination by this culture was supported by supplementation with 0.05 mg/l vitamin B_{12}, 25% v/v anaerobic digestor sludge supernatant (SS), and 2 mM acetate, which

presumably served as a carbon source. Neither methanol nor acetate could serve as an e^- donor for dechlorination by the H_2/PCE culture, and it did not produce CH_4 or acetate from H_2/CO_2 or methanol, indicating the absence of methanogenic and acetogenic bacteria. Microscopic observations of the purified H_2/PCE culture showed only two major morphotypes: irregular cocci and small rods.

2.2 INTRODUCTION.

Several studies have been performed on an anaerobic enrichment culture which uses methanol as the electron (e^-) donor for reductive dechlorination of PCE to ETH. In initial studies (9), PCE was fed every two days at a dose of 3.5 μmol/l of culture medium. VC was the primary product, and most of the 0.32 mmol/l of methanol that was added as an e^- donor was used for methanogenesis. In a subsequent study (6), PCE and methanol doses were gradually increased until they reached 0.55 mmol/l PCE and 1.6 mmol/l methanol. This increase drastically improved the performance of the culture, with nearly complete conversion of PCE to ETH occurring within four days. The culture also had essentially ceased producing methane from methanol and, instead, the reducing equivalents from methanol not used for PCE reduction were used for acetogenesis.

More recent time-course studies (23) have shown that the methanol/PCE culture was capable of reductive dechlorination of all chloroethenes. PCE was initially degraded quantitatively to VC. VC dechlorination to ETH did not usually commence until other

chloroethenes were absent and VC dechlorination showed first-order kinetics. The culture also made small amounts of methane, usually after PCE depletion.

While methanol could serve as e^- donor for the culture, and appeared to be especially well-suited for sustained dechlorination, it was found in early studies that glucose, formate, and H_2 could also serve as e^- donors (9). Studies with other cultures have shown that H_2 (14), fatty acids (12) and even toluene (21) could stimulate PCE dechlorination. It was possible that some of these e^- donors were serving as sources of a more universal e^- donor, H_2. For the methanol/PCE culture, H_2 could readily replace methanol for several feedings (5), although eventually performance faltered. Good performance was restored by resuspension of the H_2/PCE culture in the supernatant from the methanol/PCE culture, suggesting that the latter contained growth factors required for dechlorination. The eubacterial cell wall synthesis inhibitor vancomycin inhibited PCE dechlorination and acetogenesis from methanol, but PCE dechlorination from H_2 was not affected. This suggested that the PCE dechlorinator was resistant to vancomycin, and that it could not use methanol directly, but rather, required methanol metabolism, most likely to provide H_2 or formate as the e^- donor for reductive dechlorination.

Fig. 2.1 shows a model of the metabolism of methanol and PCE by the dechlorinating culture which includes the hypotheses that H_2 is the actual e^- donor for dechlorination, and that there is a nutritional contribution made to PCE-dechlorinators by methanol-me-

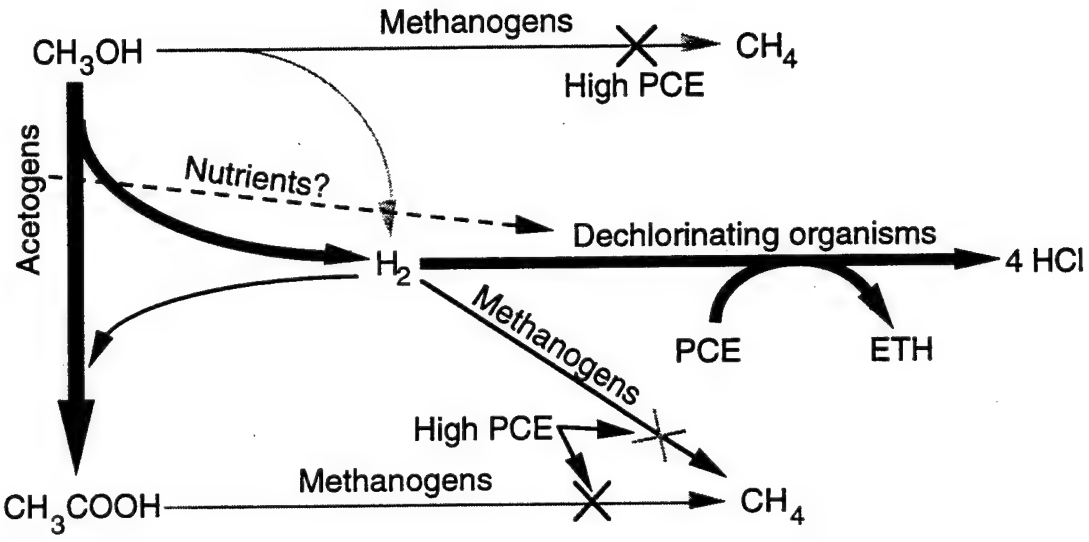

FIGURE 2.1. Model, based on DiStefano *et al.* (5) for carbon and e^- flow in a methanol/PCE anaerobic mixed culture.

tabolizing organisms (4, 5). This model suggests that a better-defined culture on H_2 and PCE could be obtained if its nutritional needs are met. In this chapter, we examine the microbial populations in the methanol/PCE culture, and describe the characterization of a highly purified H_2/PCE culture derived from a 10^{-6} dilution of the methanol/PCE culture.

2.3 MATERIALS AND METHODS.

2.3.a Chemicals.

High performance liquid chromatography (HPLC)-grade PCE (Aldrich Chemical Co., Inc., Milwaukee, Wis.) and methanol (Fisher Scientific, Springfield, N.J.) were used as culture substrates. H_2 was purchased from Empire Airgas, Inc., Elmira, N.Y. VC was obtained as a gas from Matheson Gas Products, Inc., Secaucus, N.J. Other chloroethenes were obtained from Aldrich Chemical Co. Ethane, ethene, and methane were purchased from Supelco, Bellefonte, Penn. All other chemicals used were reagent grade or better.

2.3.b Analyses.

For qualitative analysis of ethenes in most probable number tubes, a Model 1400 flame ionization detector-gas chromatograph (GC) (Varian, Walnut Grove, Calif.) was used with a 2 m x 3 mm stainless steel column packed with 60/80 mesh Carbopak B/1% SP-1000 (Supelco, Bellefonte, Penn.) and operated isothermally at 200° C

as described previously (23).

For quantitative analysis of chloroethenes and ETH, gas (headspace) samples of 100-µl were analyzed using a Model 8610 GC (SRI Instruments, Las Vegas, Nev.) equipped with flame ionization detector and using the same column and operating conditions as described above. Peak areas were calculated using the Peaksimple 2 software supplied with the GC, and were compared to standard curves for chloroethenes (13). Partition coeficients for the different compounds, as obtained by Gossett (13) and Freedman (8) at 35°C in serum bottles with an aqueous volume of 100 ml and a headspace volume of 60 ml were (in parenthesis, the compound partitioning in the aqueous phase in %): PCE=1.18 (58.5); TCE=0.63 (72.6); *cis*-DCE=0.235 (87.6); *trans*-DCE=0.575 (74.3); 1,1-DCE=1.52 (52.3); VC=1.38 (54.7); and ETH=9.06 (15.5). When we added 5 µmol PCE to a 27 ml crimp-top tube containing 10 ml of growth medium, the nominal concentration was 0.5 mmoles/liter, while the estimated aqueous concentration (13) was 0.16 mmol/liter.

When methane and ETH were produced together, they were measured with a Varian 1400 GC using a Porapak R column as described previously (18). The detection limit for methane was ca. 0.1 Pa, or 1 nmol/tube. H_2 was analyzed by gas chromatography with a AGC 110 thermal conductivity GC (Hach Carle, Lehigh Valley, Penn.) using N_2 as the carrier gas as described previously (28). Headspace samples were 100-µl in all cases. Acetate concentrations were quantified using HPLC with a fast acid analysis column (Bio Rad, Richmond, Calif.) and a solvent of 6.5 mM H_2SO_4 as previously

described (30). Total organic carbon was measured using a Model 5010 organic carbon analyzer (UIC Inc., Joliet, Ill.).

2.3.c Growth medium and culture conditions.

Methanol/PCE cultures were incubated in the growth medium and under conditions described previously (23). Unless stated otherwise, the basal medium contained (in g/l final concentration): NH_4Cl, 0.5; K_2HPO_4, 0.4; $MgCl_2 \cdot 6H_2O$, 0.1; $CaCl_2 \cdot 2H_2O$, 0.05; resazurin, 0.001, and trace metal solution (27), modified by addition of 0.01 g/l $NiCl_3 \cdot 6H_20$), 10 ml/l. The medium was purged with N_2 and dispensed inside an anaerobic chamber into 27 ml crimp-top tubes (Bellco, Vineland, N.J.). The tubes were then sealed with aluminum-crimp Teflon-coated butyl rubber stoppers (Wheaton, Millville, N.J.) and autoclaved at 121°C for 55 min. The tube headspaces were flushed with sterile 70%N_2-30%CO_2 (Matheson Gas Products, Inc., Secaucus, N.J.), and received the following sterile and anaerobic additions before inoculation (final volume ≈ 10 ml/tube): $Na_2S \cdot 9H_2O$, 2 mM; $NaHCO_3$, 12 mM; anaerobic digestor sludge supernatant (SS), 25% v/v; yeast extract (YE), 0.2 g/l or sodium acetate, 2 mM; and vitamin solution (1) concentrated 10-fold, 0.5% v/v.

The anaerobic sewage digestor sludge for preparation of SS was obtained from the Ithaca Area Wastewater Treatment Facility. Several liters were collected at one time, to assure uniformity. The sludge was initially clarified by centrifugation for 30 min. at 8,000 RPM (10,400 x g) using a Sorvall RC-2B centrifuge (DuPont-Sorvall, Wilmington, Del.). Samples were stored frozen at -20° C until use. Thawed samples

were centrifuged for 30 min. at 18,000 RPM (39,000 x g). The resulting supernatant was neutralized to pH 7.0 using 1 M HCl, and then prefiltered through a 0.8 μm Supor-800 membrane filter (Gelman Sciences, Ann Arbor, Mich.). The SS was then purged with N_2 and filter-sterilized in an anaerobic chamber through a double 0.8/0.2 μm Acrodisc PF filter (Gelman) into sterile vials. In some instances, the SS was lyophilized in a Labconco LYPH·LOCK 4.5 liter Freeze Dry system, model 77510 (Labconco Corporation, Kansas City, Mo.).

Unless otherwise stated, inoculum sizes were 2% v/v, all incubations were done in duplicate, and each experiment presented was performed at least twice with similar results. Duplicate tubes performed very similarly in the experiments presented (less than 5% difference in results for tubes under same conditions), so some results are presented for individual tubes. Cultures that used H_2 as e^- donor were incubated upside down in the dark in a R76 incubator-shaker (New Brunswick Scientific, Edison, N.J.) operated at 35°C and 150 rpm. H_2 was added to the headspace as over-pressure (0.67 atm, 67 kPa, or ca. 47.5 mmol/l) immediately after inoculation. $NaHCO_3$ was added to neutralize the HCl produced by the dechlorination process (23). The quantity of PCE added to a culture was estimated directly from the delivered syringe volume (6).

2.3.d Most probable number (MPN) determinations of the methanol/PCE culture.

Three-tube MPN determinations were performed for a variety of microbial groups present in the methanol/PCE culture. All tubes

contained the basal medium described above. A master set of dilutions was made in 160 ml serum vials containing 45 ml of basal medium, which was used for all subsequent dilutions. H_2/CO_2-utilizing methanogens and acetogens were enumerated using medium to which H_2 was added at 130 kPa (ca. 90 mmol/l) after inoculation. Methanol-utilizing methanogens and acetogens were enumerated using medium to which 24 mM methanol was added, while 40 mM Na acetate was used for culturing acetate-utilizing methanogens. Sulfidogens (e.g. sulfate reducers) were enumerated using medium which received 5 mM Na acetate, 130 kPa H_2, and 10 mM $Na_2S_2O_3$, which is used by more sulfidogens than sulfate (26). Fermentative heterotrophs were enumerated in medium to which an additional 1 g/l YE and 0.25 g/l each of glucose, cellobiose, xylose, and sucrose were added. Tubes were considered positive when greater turbidity was observed than in control dilutions lacking these additions and when the appropriate products (methane or acetate) were detected. Methanol-utilizing PCE dechlorinators were enumerated in medium to which 12 mM methanol was added together with the equivalent of 0.2 mmol/l of PCE. Cultures were considered positive if they produced greater than trace quantities of reduction products (generally, VC and ETH were the only products detected). H_2-utilizing PCE dechlorinators were enumerated and assayed in a similar manner, except that 130 kPa H_2 replaced methanol as the e^- donor.

2.3.e Microscopy.

A Zeiss Standard 18 microscope was used for phase-contrast ob

servation of cells and for epifluorescence microscopy to detect organisms with characteristic cofactor F_{420} autofluorescence typical of most methanogens as described previously (29). For photomicroscopy, cells were spread out on an agar-coated slide and were stained with 0.01% acridine orange. The cells were observed using a Zeiss laser scanning microscope (model LSM-10) using either phase-contrast or epifluorescent (488 nm) imaging.

2.4. RESULTS.

2.4.a Viable counts of microbial populations in the methanol/PCE culture (these studies were conducted by V. Tandoi).

The most probable number (MPN) technique was used to estimate the numbers of members of various microbial groups in the methanol/PCE culture (Table 2.1). Neither methanol- nor acetate-utilizing methanogens were detected in the culture. A relatively low number of H_2/CO_2-utilizing methanogens were detected, and microscopic examination of the highest positive dilution tubes showed spirals with the distinctive morphology of *Methanospirillum*, a methanogen capable of using either H_2/CO_2 or formate (1). High numbers of methanol-utilizing acetogens were found. A preliminary characterization of these chain-forming cocci indicated that they stained Gram-positive, required SS for growth, could not use H_2/CO_2 for growth, and did not dechlorinate PCE using either methanol or H_2 as the e^- donor (24). The numbers of H_2/CO_2-utilizing acetogens were

TABLE 2.1 Most probable number (MPN) determinations for various microbial populations in a methanol/PCE culture (these studies were conducted by V. Tandoi). The abbreviation "util." stands for "utilizing".

Organism type	MPN (ml^{-1})	Time (wks) until highest dilution was positive	Predominant morphology in highest positive dilution
H_2/CO_2-util. methanogens	2.3×10^4	5	Long spirals
Methanol-util. methanogens	≤2.3	-	-
Acetate-util. methanogens	≤2.3	-	-
H_2/CO_2-util. acetogens	$\leq 2.3 \times 10^4$	-	-
Methanol-util. acetogens	9.3×10^6	2	Cocci in chains and doublets
H_2-util. thiosulfate reducers	9.3×10^5	2	Small spirals
Fermentative heterotrophs	2.3×10^7	2	Various rods
Methanol-util. PCE dechlorinators	2.3×10^3	5	-
H_2-util. PCE dechlorinators	2.3×10^6	5	Small and large rods, irregular cocci and some small spirals

lower than those for methanogens. There were relatively high counts of sulfidogens and the predominant morphotype in the highest positive dilution tubes resembled desulfovibrios. The highest MPN counts were for fermentative heterotrophs capable of growing in medium containing YE and sugars.

In MPN dilution tubes receiving methanol and PCE, the highest dilution which showed products of reductive dechlorination was 10^{-3}, in which VC was detected within 3 weeks. There was growth in the methanol/PCE tubes out to 10^{-7} dilutions, and chain-forming cocci similar to those seen in tubes receiving methanol without PCE were found in these tubes.

In MPN dilution tubes receiving H_2 and PCE, serial dilutions became positive for VC production sequentially until, after 5 weeks of incubation, two of three 10^{-6} tubes were positive for VC production, with the third tube becoming positive approximately 1 week later. Although analysis of PCE and its products was qualitative, VC was the only daughter product initially detected in positive tubes until PCE was depleted, at which time ETH was detected. Microscopic examination of these cultures revealed small and large rods, individual irregular cocci, and *Desulfovibrio*-like spirals. These 10^{-6} dilution tubes consumed several subsequent doses of PCE, and transfers from these cultures were the source of the purified H_2/PCE cultures described in the rest of this communication.

2.4.b PCE utilization by the purified H_2/PCE culture.

Fig. 2.2 shows that after the H_2/PCE culture was transferred

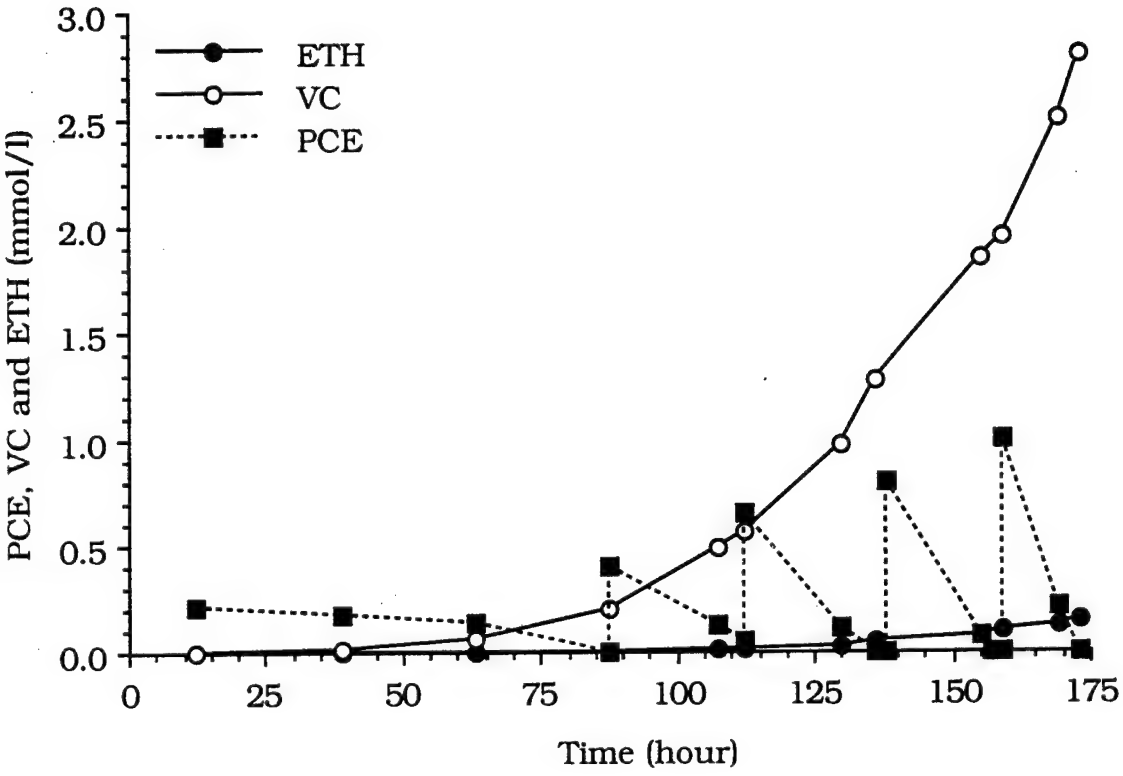

FIGURE 2.2 PCE dechlorination and product formation by the purified H_2/PCE culture to which consecutively increasing PCE doses were added. The basal medium was amended with 25% (v/v) SS, a vitamin solution and 2 mM Na acetate {inoculum size was 2% (v/v)}.

into fresh medium, the rate of PCE utilization increased over time, indicating growth of the PCE-dechlorinating organisms. The major dechlorination products detected were VC and ETH, which were produced nearly stoichiometrically from PCE. There was always some loss of chloroethenes into or through the stoppers. Fig. 2.3 shows the results of a set of controls performed for PCE, VC and ETH to ascertain the extent of ethene loss into and/or through the stopper. It was observed that the more chlorinated an ethene was, the greater the proportion lost (results for *cis*-DCE and TCE are not shown).

As shown in Fig. 2.2, by day 6 the culture was able to consume the equivalent of 1 mmol PCE added per liter of culture medium in 15 hours. Table 2.2 shows that cultures not provided with H_2 did not dechlorinate PCE and that methanol did not serve as an e^- donor for PCE dechlorination. In cultures provided with PCE, there was a greater disappearance of H_2 than in cultures lacking PCE or an inoculum, and the net amount consumed, 4.7 mmol/l, was approximately equal to the amount required to reduce 1.66 mmol of PCE per liter to VC (4.9 mmol/l). Moreover, cultures provided with H_2 or methanol in the presence or absence of PCE produced neither methane (data not shown) nor acetate (Table 2.2), indicating the complete absence of methanogens and acetogens, while in dechlorinating cultures the initial acetate concentration decreased as PCE was being consumed.

2.4.c Nutritional characterization of the H_2/PCE culture.

The H_2/PCE culture could be transferred indefinitely in medium

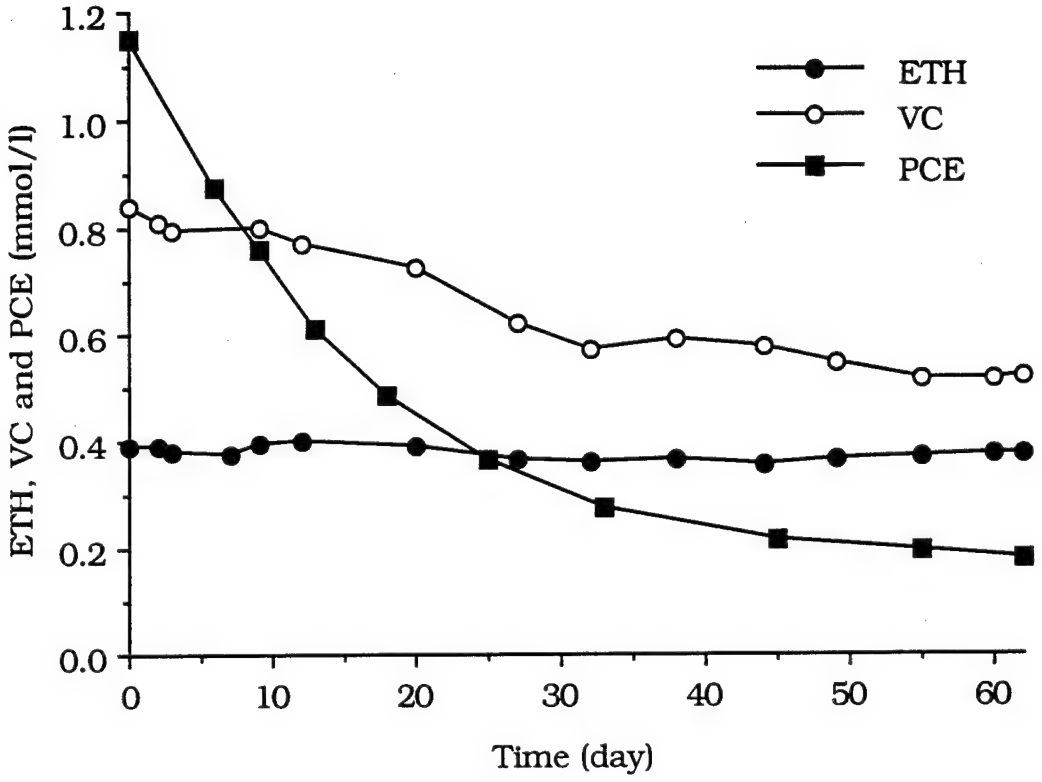

FIGURE 2.3 Loss of PCE, VC and ETH into and/or though the stopper. Results for PCE were averaged from three sets of duplicate tubes under the next conditions: i). Not inoculated tubes with all other amendments; ii). not inoculated tubes with all other amendments but the vitamin solution; iii). tubes inoculated and autoclaved (standard deviations were very small and have not been plotted for simplicity).

TABLE 2.2 Effect of e^- donors on PCE dechlorination (mainly to VC and ETH) and acetogenesis by the purified H_2/PCE culture.

Additions	Products (mmol/l)[a] on Day 14	H_2 decrease (%)[b] on Day 14	Acetate (mM)[c] Day 1	Day 14
H_2 + PCE	1.66	10.1	1.97	1.60[d]
MeOH + PCE	0	NA[e]	1.94	2.03
No e^- donor + PCE	0	NA	2.04	2.06
No e^- donor (no PCE)	NA	NA	1.97	1.98
H_2 (no PCE)	NA	3.3	2.06	1.94
MeOH (no PCE)	NA	NA	1.97	1.97
H_2 (no PCE; no inoc.)	NA	3.2	2.07	2.06

[a] Values obtained from PCE dechlorination to almost completely ETH and VC.

[b] Percentage obtained from the mean of duplicate tubes (sampling headspace). Standard deviations are 1.5 for the H_2/PCE cultures; and 1.0 for the H_2 (no PCE) and H_2 (no PCE; no inoc.) cultures.

[c] Mean concentration of duplicate tubes (sampling liquid phase). Standard deviations were 0.3 for the H_2/PCE cultures and 0 to 0.1 for the rest of the cultures.

[d] Value measured after 6 days, when 0.23 mmol/l of products had been produced.

[e] na = Not applicable.

containing 0.2 g/l YE, 25% v/v SS, and a vitamin solution as nutritional supplements. It was desirable to replace YE with nutrients which would support less growth of contaminants while still supporting PCE dechlorination.

Our initial studies showed that deleting YE from the growth medium did not allow sustained PCE dechlorination (data not shown). Since it was possible that YE or some product derived from it was serving as a carbon source, we tested whether YE could be replaced with acetate, a common carbon source used by many anaerobes, but which is not utilizable as an energy source by most anaerobes. Fig. 2.4 shows that acetate greatly stimulated dechlorination by the H_2/PCE culture when YE was not present. Formation of dechlorination products (almost completely VC and ETH) essentially stopped after day 5 in cultures grown without acetate, while they continued to increase rapidly when 2 mM Na acetate was present. Acetate concentrations of 10 mM were somewhat inhibitory to PCE dechlorination (data not shown). Cultures could be transferred indefinitely with 2 mM sodium acetate (replacing YE), 25% v/v SS, and vitamins. The MPN of YE-utilizing fermentative heterotrophs in this medium was 2.3×10^6, a tenfold reduction compared to cultures receiving YE, such as the methanol/PCE culture (Table 2.1).

In testing dechlorinating activity under different combinations of YE, SS and acetate, in the presence of vitamins adding acetate alone essentially allowed no PCE dechlorination, while PCE dechlorination occurred in the presence of both, acetate and SS,

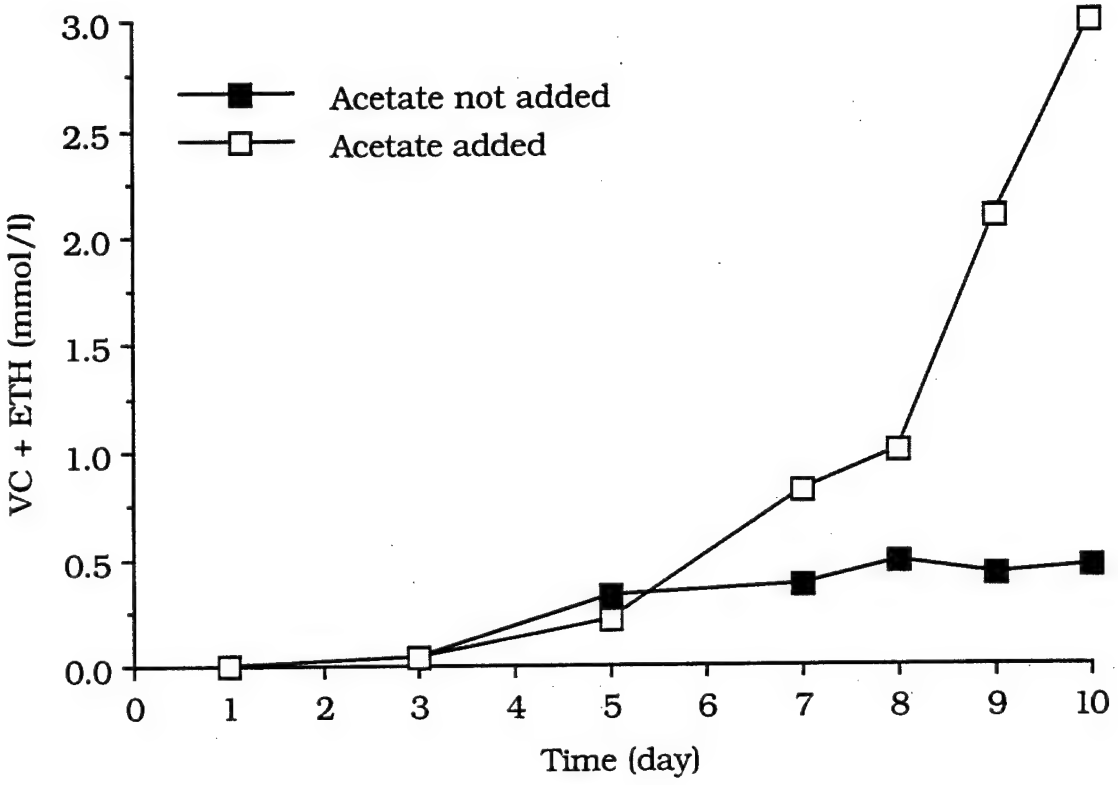

FIGURE 2.4 Product formation (predominantly VC and ETH) from PCE dechlorination by the purified H_2/PCE culture in the presence or absence of 2 mM Na acetate. The basal medium was amended with 25% (v/v) SS and vitamins and the inoculum was 1% (v/v).

implying the requirement by the culture for the latter amendment (data not shown). Even though cultures grown on YE+SS generated more PCE products than cultures grown on acetate+SS, the latter conditions gave a "cleaner" degradation process, in which less intermediate products (TCE and DCEs) and more ETH were formed (data not shown). Cultures grown on SS or YE only started dechlorinating normally but, after the second dose of PCE, they slowed down and accumulated intermediates, indicating limitation (data not shown). This experiment reinforced the importance of both acetate and SS in the nutrition of the culture. Uninoculated controls with SS and/or vitamins did not dechlorinate PCE.

The potential requirements for vitamins and SS were also examined for the H_2/PCE culture. The experiment shown in Fig. 2.5 revealed that there was limitation of dechlorination in the cultures not receiving vitamins when compared to cultures that received the vitamin solution. After vitamins were added at day 16 (Fig. 2.5 b), the culture dechlorinated PCE essentially as the positive control (Fig. 2.5 a). To identify which vitamins were required, an experiment was performed in which one of the ten vitamins present in the standard solution was deleted from each set of duplicate tubes (Fig. 2.6). It was found that cultures with vitamin B_{12} deleted showed as poor dechlorination as cultures receiving no vitamins, while deleting the other vitamins had negligible effects in products formation (with the exception of riboflavin, which showed an intermediate response). The total rates of product formation were calculated over 18 to 26 days, depending on the speed of the culture.

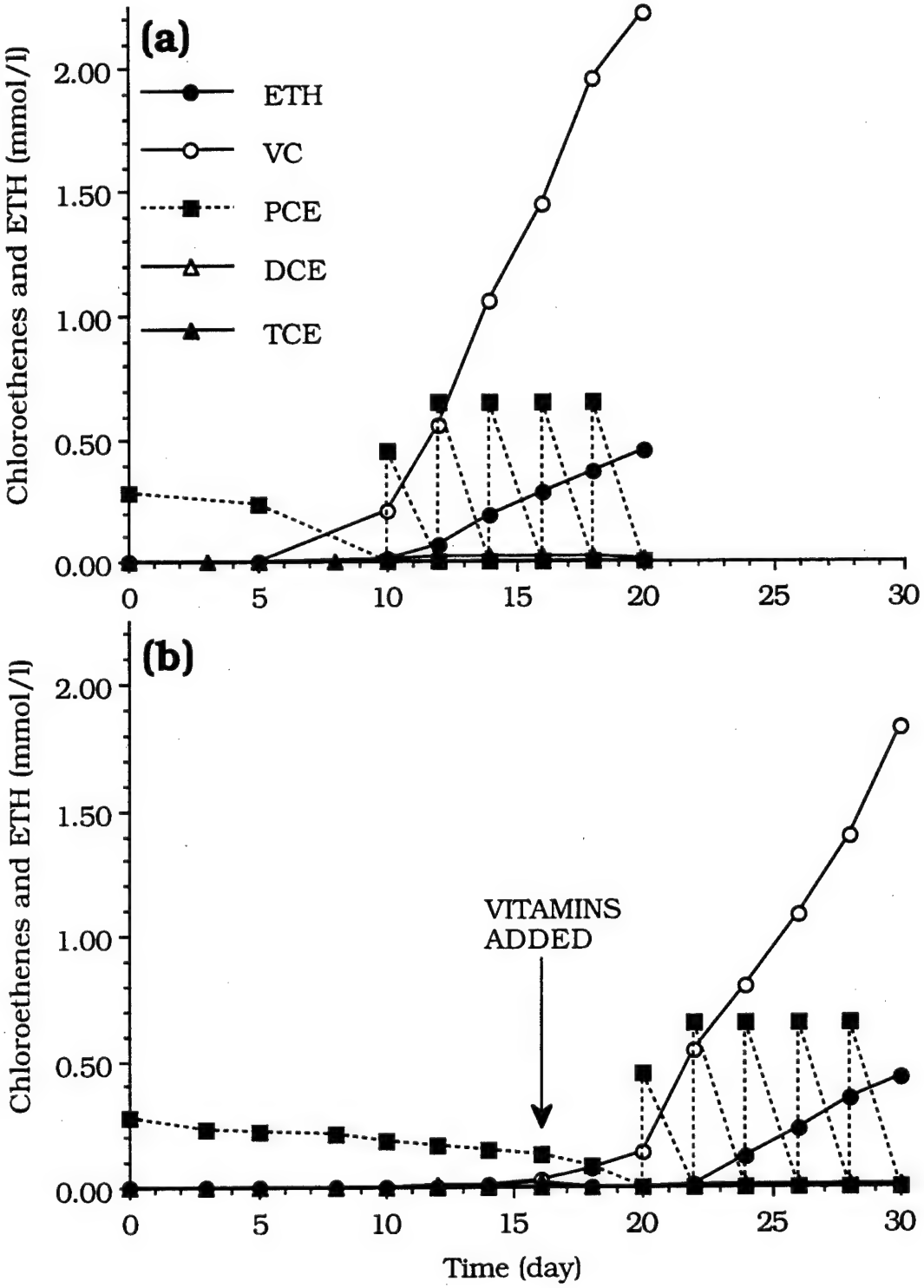

FIGURE 2.5 PCE dechlorination and ETH and VC formation by the purified H_2/PCE culture in the presence **(a)** and absence **(b)** of the vitamin solution.

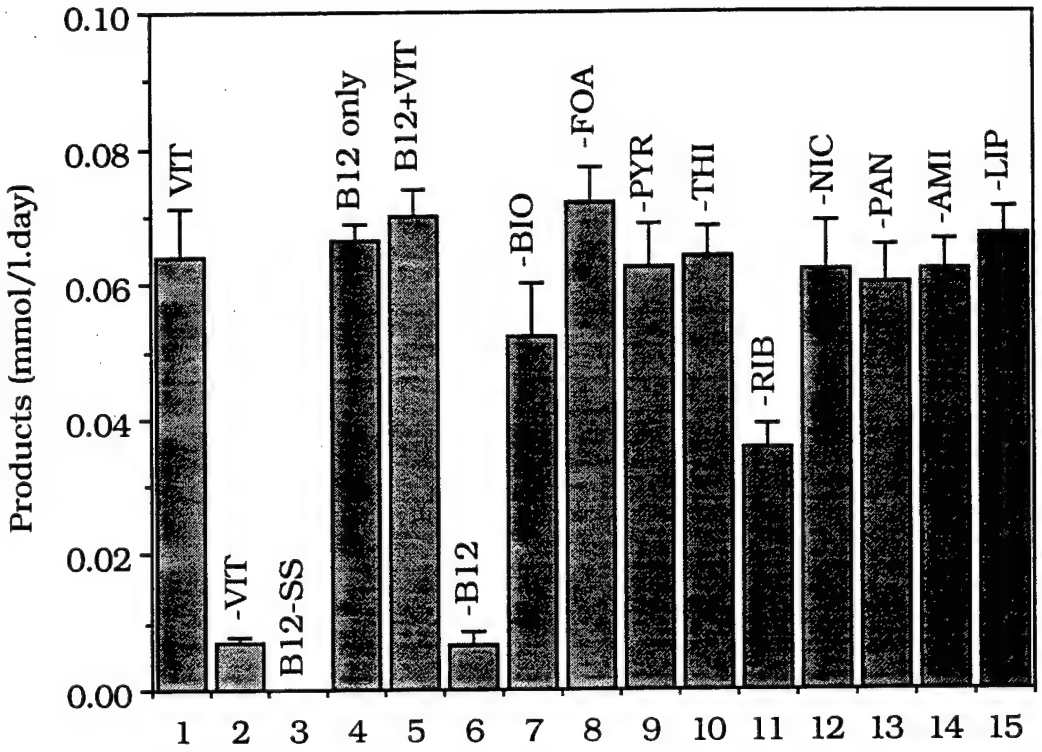

FIGURE 2.6 Product formation (TCE, DCEs, VC and ETH) by cultures incubated under different vitamin treatments: column 1(VIT)=vitamin solution present (positive control); 2 (-VIT)=vitamin solution absent (negative control); 3 (B_{12}-SS)=cyanocobalamin present but without SS; 4 (B_{12} only)=only cyanocobalamin present; 5 (B_{12}+VIT)=all vitamins present with a double amount of B_{12}; 6 (-B_{12})=all vitamins present except B_{12}; 7 (-BIO)=all vitamins present except biotin; 8 (-FOA)=all vitamins present except folic acid; 9 (-PYR)=all vitamins present except pyridoxine hydrochloride; 10 (-THI)=all vitamins present except thiamine hydrochloride; 11 (-RIB)=all vitamins present except riboflavin; 12 (-NIC)=all vitamins present except nicotinic acid; 13 (-PAN)=all vitamins present except calcium pantothenate; 14 (-AMI)=all vitamins present except p-aminobenzoic acid; and 15 (-LIP)=all vitamins present except lipoic acid.

The requirement for vitamin B_{12} was then examined more closely. Fig. 2.7 shows the effect of adding various amounts of vitamin B_{12} on product formation (mainly VC and ETH) by the culture after 11 days of incubation (no other vitamins were present). Increasing amounts of vitamin B_{12} led to increasing product formation, although the response was not linear. Saturation occurred near 0.05 mg/l vitamin B_{12}, at which concentration the cultures performed as well as ones receiving all ten vitamins. In uninoculated cultures, vitamin B_{12} added to concentrations up to 5 mg/l did not catalyze measurable reductive dechlorination of PCE (data not shown).

Studies were also performed on the SS, which contained 75 mg/l organic carbon and $\leq$ 0.05 mM acetate or other volatile fatty acids. As seen in Fig. 2.8, when SS was not added to the growth medium, the culture did not consume the first dose of PCE within 14 days, suggesting nutrient limitation. Adding the lyophilized equivalent of 5% v/v SS allowed the consumption of four increasing doses of PCE, with little accumulation of ETH and meaningful accumulation of DCEs and TCE between feedings (preliminary tests showed that lyophilized SS performed as well as SS; data not shown). Adding the equivalent of 25% v/v SS, the standard dose used in other experiments, allowed the consumption of multiple and increasing doses of PCE, although there were signs of limitation (residual TCE and PCE) after the last dose. Addition of 50% v/v SS allowed the best consumption of PCE, with the greatest ETH accumulation, while cultures amended with 100% v/v SS did not perform as well, showing a decrease in the rate of PCE degradation at the end of incubation.

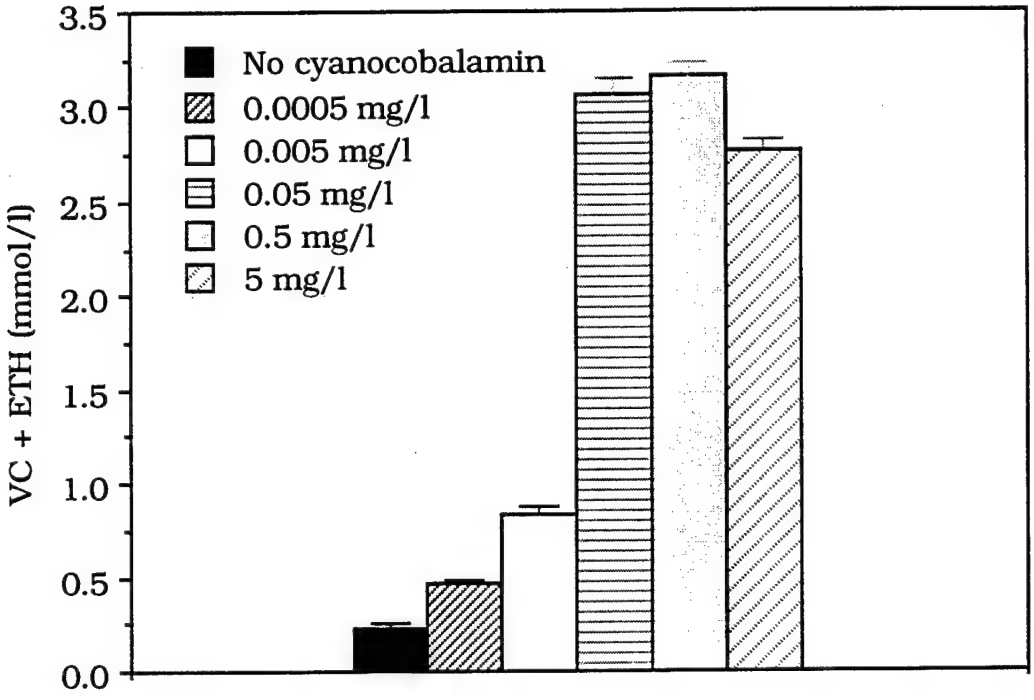

FIGURE 2.7 PCE dechlorination products as measured after 11 days from inoculation (1% v/v) of the purified H_2/PCE culture in tubes containing different concentrations of vitamin B_{12}. The basal medium was amended with 25% v/v SS and 2 mM Na acetate.

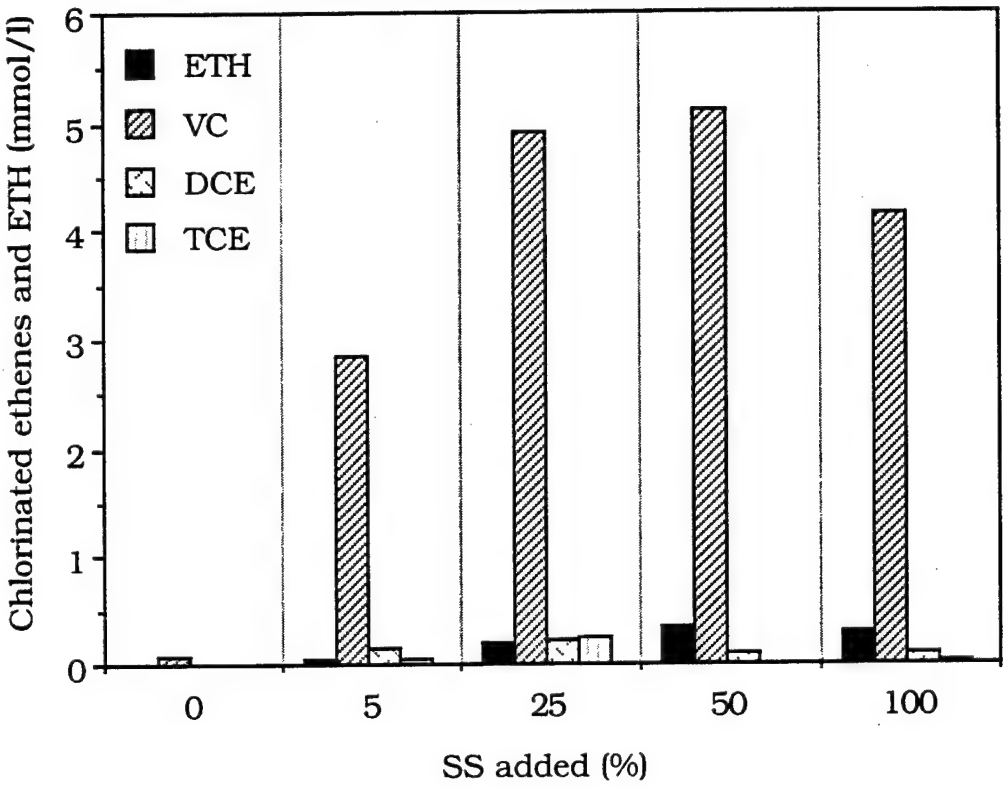

FIGURE 2.8 PCE dechlorination products as measured after 14 days from inoculation (2% v/v) of the purified H_2/PCE culture. Tubes were amended with different percentages of lyophilized SS and were given new PCE doses as soon as PCE was consumed. The basal medium was amended with vitamins and 2 mM Na acetate.

2.4.d Microscopic observations of the methanol/PCE culture and the purified H_2/PCE culture.

In the purified H_2/PCE culture grown in medium with acetate, SS, and vitamins, the two major morphotypes were irregular cocci and small rods (Fig. 2.9). Larger rods were also readily observed, but were far less numerous than the previously mentioned morphotypes. The irregular cocci were absent from cultures which did not show PCE dechlorination (data not shown), suggesting their involvement in that process, although more conclusive evidence is needed before ascribing the process to any one organism.

2.5 DISCUSSION.

The results presented here are consistent with the primary prediction of the model of DiStefano *et al.* (5) that H_2 (or possibly formate) is the primary e^- donor for reductive dechlorination in the methanol/PCE culture (Fig. 2.1). Specifically, the prediction was borne out that there should be relatively high numbers of methanol-utilizing acetogens and H_2-utilizing PCE dechlorinators in the culture. Indeed, when tested after several transfers, the H_2/PCE culture derived from a 10^{-6} dilution of the methanol/PCE culture was no longer capable of using methanol as an e^- donor for dechlorination or for acetogenesis (Table 2.2). Presumably, the methanol-utilizing acetogens were initially present in the 10^{-6} H_2/PCE dilutions, since they were present in the original methanol/PCE culture at numbers exceeding 10^7/ml, but were lost after several transfers in medium

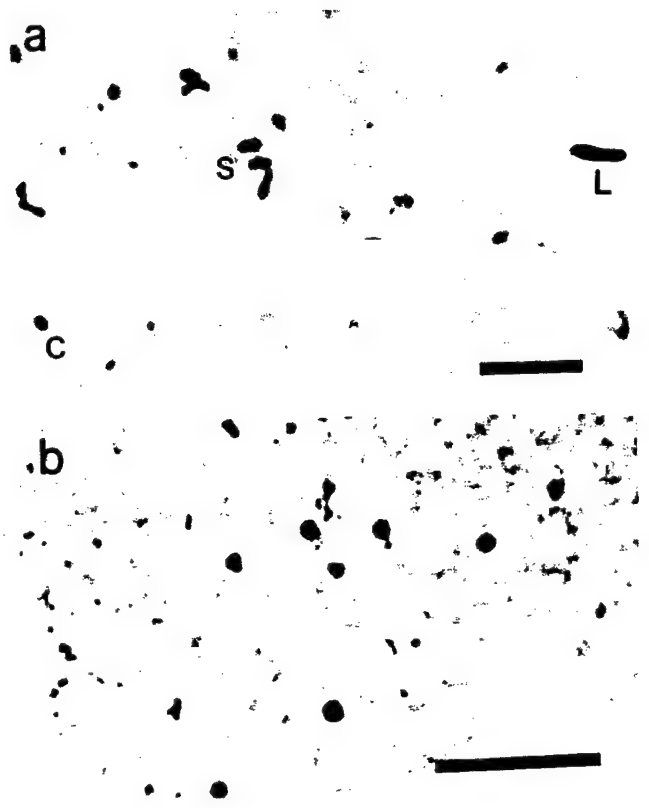

FIGURE 2.9 (a) Photomicrograph of the purified H_2/PCE culture concentrated 25-fold showing short rods (S), long rod (L) and irregular cocci (C). **(b)** Higher magnification photomicrograph of the concentrated H_2/PCE culture showing a microscopic field containing many irregular cocci. The microscopy conditions were adjusted to enhance the contrast between the cocci and the background. Marker bars represent 5 μm.

lacking a substrate for their growth. Acetate, present at 2 mM, also could not serve as an e^- donor for dechlorination (Table 2.2).

The model also predicts low numbers of methanogens in the methanol/PCE culture, and only a low number of H_2/CO_2 utilizing methanogens were detected (Table 2.1). This is in contrast to earlier studies on the methanol/PCE culture when the PCE dose was much lower and the culture was actively methanogenic (9), in which we found over 10^7/ml of a methanol-utilizing methanogen resembling *Methanosarcina* (29). The absence of methanogens and acetogens in the purified H_2/PCE culture indicates that these organisms were not primarily responsible for the high-rate PCE dechlorination in the original methanol/PCE culture.

H_2 was able to serve indefinitely as the e^- donor for reductive dechlorination of PCE to VC and ETH in cultures provided with proper nutritional supplements, in agreement with previous results (5). The degradation of PCE by the H_2/PCE culture at an increasing rate as well as our ability to transfer it indefinitely is indicative of growth of the culture concurrent with the dechlorination process. H_2 and PCE have been shown to serve as an e^- donor/acceptor pair for the growth of several cultures (11, 14, 16, 20, 22), but in those cases, reductive dechlorination stopped at *cis*-DCE. No evidence was obtained for DCE accumulation in this culture and neither of the two dominant morphotypes in the purified H_2/PCE culture, when examined microscopically, resembled any of the species isolated so far. It is then likely that a different organism is responsible for PCE dechlorination in this culture.

That a 10^{-6} dilution of the methanol/PCE culture on H_2/PCE required thirty-five days (Table 2.1) to accomplish approximately 20 doublings ($2^{20} \approx 10^6$) suggests a doubling time less than 2 days for the H_2/PCE culture, a doubling time consistent with the increasing rate of metabolism in Fig. 2.2.

The purified H_2/PCE culture was able to convert PCE to ETH, although the ratio of ETH/VC produced from PCE was much lower than that described for the methanol/PCE culture (6). This is at least partially due to the feeding regime used for the H_2/PCE culture in these experiments, in which we provided a dose of PCE as soon as its depletion was detected. Since PCE can inhibit VC dechlorination (23), the cultures had little opportunity to accumulate ETH.

The purified H_2/PCE culture could be transferred indefinitely in growth medium supplemented with acetate, SS, and vitamins. The acetate most likely serves as a carbon source for the culture, and as such, supports considerably less growth of potential contaminants than does YE. The optimal amount of SS required was 25-50% v/v. It is not clear whether the SS directly provides nutrients required by PCE dechlorinators, or whether products of metabolism of SS components by other organisms are required. The SS contributed less than 20 mg/l of carbon to the culture when added at 25% v/v, and since some portion of it is not readily catabolizable, it should support only a low number of contaminants. The amount of acetate provided by 25% v/v SS, < 0.05 mM, was too low to support growth of the dechlorinators.

The vitamin requirement for PCE dechlorination could be met by adding vitamin B_{12} alone, but since we also needed to add SS in these experiments, it is possible that the SS was providing sufficient quantities of other required vitamins. The vitamin B_{12} requirement is intriguing because it, and other corrinoid compounds, have been shown to carry out reductive dechlorination *in vitro* of chloro-organics, including chloroethenes (10, 15, 19). We verified that no reductive dechlorination of PCE occurred in uninoculated media supplemented with vitamin B_{12}. Still, the requirement for vitamin B_{12} suggests a role in dechlorination in this organism, perhaps as a prosthetic group of a dechlorinating enzyme. The amount required, between 0.005 mg/L and 0.05 mg/L, is considerably greater than the amount (0.001 mg/L) typically supplied to organisms which use vitamin B_{12} for anabolic reactions (2). The vitamin B_{12} requirement may explain why methanol was the e^- donor which led to the best sustained dechlorination in the early studies on this culture (6, 9). Those cultures were amended with YE, which lacks vitamin B_{12} (2). However, methylotrophic methanogens (3) and acetogens (17) growing on methanol are rich in cobamides, which could be cross-fed to the dechlorinating organisms by lysis and possibly excretion. For example, a *Methanosarcina* culture, grown on acetate, supported growth of a vitamin B_{12}-requiring contaminant (25).

These results show that like many other anaerobes, the PCE dechlorinator/s in this culture are dependent on other organisms for several of their nutritional requirements. Knowledge of some of these requirements has allowed us to simplify the culture medium we use

for them, and it is hoped that further knowledge of these requirements will lead to the isolation of PCE-dechlorinator/s. Knowledge of the nutrition of PCE-dechlorinator/s can also have practical significance. Fennell and Gossett (7) have found in bioreactor studies that the methanol/PCE culture could be switched to butyrate as an e^- donor, but only when vitamins, including vitamin B_{12}, were added. Thus, addition of a simple vitamin solution has allowed a greater flexibility in e^- donor use by a PCE-dechlorinating mixed culture.

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CHAPTER THREE

ISOLATION AND CHARACTERIZATION OF A NOVEL EUBACTERIUM CAPABLE OF REDUCTIVELY DECHLORINATING PCE TO ETH

"It has long been an axiom of mine that the little things are infinitely the most important"

Sir Arthur Conan Doyle, 1859-1930
Adventures of Sherlock Holmes (1892) 'Case of identity'"

3.1 ABSTRACT.

The isolation of a pure culture which dechlorinates PCE completely to the non-toxic product ETH, called Strain 195, is described in this chapter. Isolation entailed using the antibiotic ampicillin, to which the dechlorinator was resistant, and supplementing the growth medium with extracts from mixed cultures. Growth was demonstrated in medium with H_2 and PCE as an electron donor/acceptor pair. Other electron donors tested were not utilized nor were electron acceptors other than chloroethenes and two dihaloethanes. Strain 195 is an irregular coccus. Its cell wall did not react with a peptidoglycan-staining fluorescent lectin and resembles protein S-layers of Archaea. Phylogenetic analysis of Strain 195's 16S rDNA sequence indicates that it is a eubacterium which shows no close affinity to known eubacterial groups. These findings significantly increase our knowledge of the diversity of organisms capable of reductive dechlorination, knowledge crucial to

understanding and manipulating the *in situ* dechlorination of chloroethenes.

3.2 INTRODUCTION.

As it has been shown in Chapter Two, under anaerobic conditions, both PCE and TCE can be reductively dechlorinated by mixed microbial populations (1, 10, 13, 33) and pure cultures (14, 19, 22, 26, 27) according to the reaction sequence shown in Figure 1.1. Even though the isolation of these pure cultures is relevant, up to this point there has not been any report of a microorganism that, by itself, is able to completely dechlorinate PCE to ETH (or to VC, the last intermediate in the reduction pathway). All axenic cultures known to date which use PCE as a respiratory electron (e^-) acceptor do so by effecting its partial reduction to *cis*-DCE without further dechlorination.

Even though the potential for complete anaerobic detoxification of chloroethenes exists, there needs to be a greater understanding of the organisms and factors involved in this reductive process. An isolate that was capable of completely dechlorinating a PCE molecule would be of great interest, not only because of its potential direct application to the bioremediation of contaminated sites (*in situ* or in above-ground facilities), but also because it could be utilized as a model for the study in depth of the dechlorination pathway, both at the biochemical and molecular levels.

We have studied a set of enrichment cultures which, using

methanol, H_2, or butyrate as an e^- donor, can dechlorinate doses of PCE of up to 550 μmol added per liter to ETH within 4 days (8, 9, 12, 30). As detailed in Chapter Two of this dissertation, a culture was derived from a 10^{-6} dilution of a methanol/PCE culture inoculated into medium with H_2/PCE (24). This partially-purified culture contained no methanogens or acetogens, and could be transferred indefinitely into H_2/PCE medium supplemented with a mixture (called ABSS) of 2 mM acetate, 0.05 mg/l vitamin B_{12}, and 25% v/v anaerobic digestor sludge supernatant (SS). These cultures could not be transferred if either H_2 or PCE was omitted, and the amounts of H_2 consumed were stoichiometric to PCE reductive dechlorination. This culture contained two main morphotypes: a pleomorphically shaped cell and a short rod. In this chapter, the isolation and initial characterization of Strain 195, a novel bacterium able to completely dechlorinate PCE to ETH, is described.

3.3 MATERIALS AND METHODS.

3.3.a Chemicals and analyses of chloroethenes.

PCE and its chlorinated derivatives, ETH, H_2, and all other chemicals were purchased and utilized as described in point 2.3.a of the previous chapter.

For quantitative analysis of chloroethenes and ETH, gas samples were analyzed using a Model 8610 GC (SRI Instruments, Las Vegas, Nev.) equipped with flame ionization detector. The GC

contained a 2 m x 3 mm stainless steel column packed with 60/80 mesh Carbopak B/1% SP-1000 (Supelco, Bellefonte, Penn.) and it was operated isothermally at 200° C. Peak areas were calculated using the Peaksimple 2 software supplied with the GC, and were compared to standard curves for chloroethenes (16).

Gas samples utilized for the growth experiments were analyzed using a temperature programmed Perkin Elmer GC 8500 equipped with an FID detector. The GC contained a 60m x 0.53 mm RTX-502.2 capillary column operated in splitless injection mode (3 μm film thickness) (Restek Corp., Bellefonte, PA). The carrier gas utilized was helium at a flow of 80 cm/s. Peak areas were calculated using the software supplied with the GC, and were compared to standard curves for chloroethenes (16). Headspace samples were 100-μl in all cases.

3.3.b Analysis of 16S rDNA.

The phylogenetic tree generated for the 16S rDNA sequence from Strain 195 was done by using the SUGGEST TREE maximum likelihood program provided by the Ribosome Database Project (RDP) (23) which places the organism on a customized phylogenetic tree. DNA was extracted from cells of Strain 195 using a microwave oven-detergent method (15). The sequence was amplified as a polymerase chain reaction product using the primers 27f and 1522r under standard conditions (20) followed by cloning using the Invitrogen (San Diego, CA) TA cloning kit, and sequencing using an ABI 373 analyzer operated by the Cornell Biotechnology Institute. Eight sequencing primers were used (20), including two against the vector, resulting in

only a single ambiguous base in the entire sequence. Other analyses of these sequences were performed by manually aligning Strain 195's sequence to other pre-aligned sequences from the RDP, followed by using the PHYLIP 3.5c package (11), including DNAML (maximum likelihood analysis), and DNADIST (Kimura model) coupled to either FITCH or NEIGHBOR.

3.3.c Growth medium and culture conditions.

The protocol followed to produce the basal medium, previous to the addition of the amendments, was followed as described in Chapter Two, and was utilized for both the partially-purified H_2/PCE culture and Strain 195. The basal medium received the following sterile and anaerobic additions before inoculation (final medium volume ≈ 10 ml/tube): $Na_2S{\cdot}9H_2O$, 2 mM (added last); $NaHCO_3$, 12 mM (added first); and the ABSS amendments {SS (prepared as described in Chapter Two), 25% v/v; sodium acetate, 2 mM; and vitamin solution (2) concentrated 10-fold, 0.5% v/v}.

The antibiotics ampicillin, vancomycin and tetracycline were prepared by dissolving them in Milli-Q water and purging the solutions with N_2 for 10 min. The small vials were then sealed with aluminum-crimp Teflon-coated butyl rubber stoppers (Wheaton, Millville, N.J.) and filter-sterilized in an anaerobic chamber through a double 0.8/0.2 μm Acrodisc PF filter (Gelman) into sterile vials. After being at 4°C for about 4 weeks, new solutions were made to replace the old ones.

In the growth experiment, protein was quantified utilizing the

NanoOrange Protein Quantitation Kit (N-6666) (Molecular Probes Inc., Eugene, OR). The reagent is highly sensitive and has a range of protein detection from 10 ng/ml to 10 μg/ml. Samples were added to the diluted NanoOrange reagent and heated at 95°C for 10 min. After cooling the mixtures to room temperature, their fluorescence emissions were measured directly in a fluorometer at an excitation of 485 nm and an emission of 590 nm.

Inoculum sizes were 2% v/v, all incubations were done in duplicate, and each experiment presented was performed at least twice with similar results. Duplicate tubes performed very similarly in the experiments presented (less than 5% difference in results for tubes under same conditions), so the results for some of the experiments are presented for individual tubes. Cultures were incubated and H_2 and $NaHCO_3$ were added as described in Chapter Two.

To prepare the mixed culture (H_2/PCE culture) pellet extract, grown mixed cultures were centrifuged for 20 min. at 17000 rpm. Pellets were resuspended with distilled water (10x concentration) and sonicated for 10 min. with a Branson Sonifier Cell Disruptor 200 (Branson Power Corp., IN) (output microtip at level 5, pulsed at 50% duty cycle). Sonicated cells were purged with N_2 for 10 min. and filter sterilized, in the anaerobic glove box, with an Acrodisc 0.8-0.2 μm diameter filter into an autoclaved vial with a N_2/CO_2 atmosphere. HCO_3^- and Na_2S were then added to buffer the medium and keep it anaerobic before placing it at 4°C. All steps previous to the purging with N_2 were performed aerobically.

To prepare the butyrate/PCE culture extract, cultures (12) were

centrifuged for 20 min. at 11000 rpm. Pellets were resuspended with distilled water (50x concentration) and frozen at -20°C until further use. After thawing at room temperature, solutions were passed through a French pressure cell at 20,000 lb/in^2 and centrifuged at 17,000 rpm (34,800g) for 20 min. The pellet was discarded and the supernatant was then purged with N_2 for 10 min. and filter sterilized inside the anaerobic globe box with an Acrodisc 0.8-0.2 μm diameter filter into an autoclaved vial with a N_2/CO_2 atmosphere. HCO_3^- and Na_2S were then added to buffer the medium and keep it anaerobic before being frozen at -20°C until further use. After thawing, the extract was kept at 4°C. All steps previous to the purging with N_2 were performed aerobically.

3.3.d Microscopy.

A Zeiss Standard 18 microscope was used for phase-contrast observation of cells. Cells were counted in a Petroff Hausser counting chamber using an epifluorescence Zeiss Universal microscope after staining with a final acridine orange concentration of 4 mg/l.

Analysis of peptidoglycan in cell wall structures and whole cells was performed by lysing the cells in boiling 4% SDS in 25 mM phosphate buffer pH (29), followed by heat fixation to a microscope slide and washing with 25 mM phosphate buffer pH 7.0 to remove SDS and other chemicals before staining. Preparations were then stained with 100 mg/l fluorescein-labeled wheat germ agglutinin (Molecular Probes Inc., Eugene, OR) (28). The stained cell preparations were observed using a Zeiss laser scanning microscope

(model LSM-10) using either phase-contrast or epifluorescent (488 nm) imaging.

Transmission electron microscopic (TEM) studies of Strain 195 were performed on a Phillips EM-201 transmission electron microscope operating at an accelerating potential of 80 kV. The protocol followed is detailed below and it was compiled from (4, 34) and modified according to the characteristics of Strain 195: The culture was sampled at the end of its exponential phase and centrifuged at 9000 rpm x 15 min. The pellet was resuspended in a cold freshly made solution containing: 5 ml basal salts medium; 5 ml 0.1 M Na cacodylate (0.05 M final concentration); and 2.5 ml 10% glutaraldehyde (2% final concentration). The mixture was fixed for 2 hours at room temperature and centrifuged (9000 rpm x 10 min). The fixed pellet was washed (30 min., 3 times) by resuspension in 1.5 ml Na cacodylate buffer and centrifuged again (9000 rpm x 10 min). The pellet was resuspended in 1% osmium tetroxide (OsO_4, in Na cacodylate buffer) for 1 h. at room temperature, centrifuged (9000 rpm x 10 min) and washed (20 min., 2 times) in Na cacodylate buffer. After a wash in basal salts medium, it was centrifuged again (9000 rpm x 10 min). One drop of 2% Noble agar (made with basal salts medium) was added to the pellet and mixed quickly. Once solidified, the agar block was cut into 1 mm cubes, which were suspended in 0.5% filtered aqueous uranyl acetate (0.01 g/2 ml) for 20 min. at 4°C. The agar blocks were then placed in 10% ethanol for 15 min. The fluid was replaced with 25% ethanol, then with 50% ethanol, 75% ethanol, 90% ethanol, twice, 100% ethanol, and finally with 1 ml fresh 100%

ethanol, twice, all for 15 min. One ml of Araldite-Embed 812 medium (without accelerator) was added and the solution mixed gently for 30 min. Another ml was added and the process repeated. Then the mixture was replaced with 2 ml of Araldite-Embed 812 medium (without accelerator) and mixed gently for 2 h. The mixture was replaced with 2 ml of Araldite-Embed 812 medium (with accelerator, 2% DMP 30) and again mixed gently for 4 h. Agar cubes were placed into the embedding molds with complete Araldite-Embed 812 medium and incubated at 70°C for 16 h. and at room temperature.

3.4 RESULTS.

3.4.a Isolation of Strain 195.

Several attempts performed to isolate the PCE-dechlorinator by dilution into liquid medium or using agar roll tubes were not successful. A promising finding was the production of PCE degradation products in initial lower dilutions in roll tubes (Fig. 3.1). Later on, 10^{-5} dilutions produced small amounts of VC. Production of chlorinated derivates eventually faded away, at a time when there were still numerous microcolonies of contaminating heterotrophs which could be seen by a dissecting microscope. Colonies of the dechlorinating organism were too small to be identified, and there was no success in obtaining PCE dehlorination in a dilution high enough to prevent the growth of heterotrophic contaminants.

To isolate the PCE-dechlorinator, a previous observation that

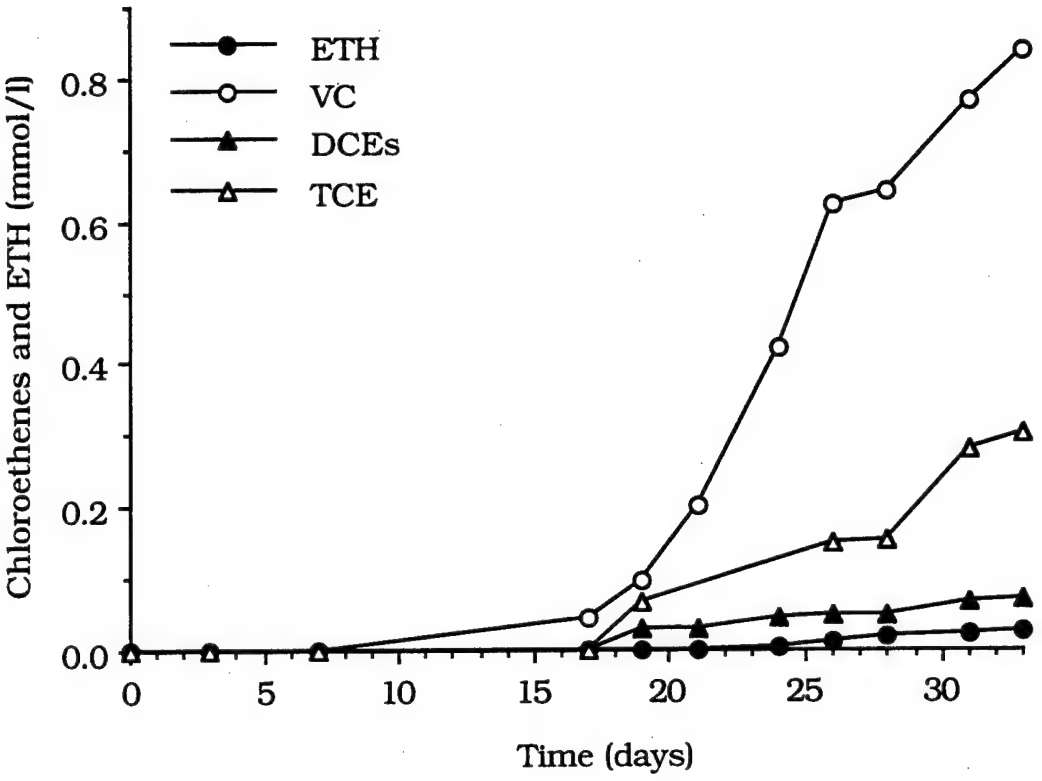


FIGURE 3.1 Conversion of PCE to less chlorinated ethenes by a purified H_2/PCE culture incubated in an agar roll tube. The tube received the equivalent of 10 µl inoculum.

PCE dechlorination using H_2 as the e^- donor was resistant to 100 mg/l vancomycin (8) (an inhibitor of eubacterial peptidoglycan cell-wall synthesis) was examined more thoroughly. It was found that the partially-purified H_2/PCE culture could be successfully transferred (2% v/v inoculum) to medium containing 100 mg/l vancomycin, up to 3 g/l ampicillin or penicillin (also peptidoglycan synthesis inhibitors) (data not shown). No PCE dechlorination was detected in cultures transferred to medium containing 20 mg/l tetracycline (a eubacterial protein synthesis inhibitor). Fig. 3.2 shows the product formation from PCE by cultures in medium containing ampicillin, vancomycin or tetracycline. In cultures that grew, only the coccoid morphotype was present at day 9 under the phase contrast microscope.

Ampicillin- or vancomycin-treated cultures could not be transferred a second time into antibiotic-containing medium. Based on previous results (8), it was suspected that other organisms in the culture were providing a nutrient(s) required by the PCE-dechlorinator and not present in ABSS. Reasoning that either the growth medium (supernatant fraction after centrifugation) or the cells (pellet fraction) in a mixed culture uninhibited by ampicillin would contain the needed factors, the ABSS medium of second-generation ampicillin-treated cultures was amended with either the filter-sterilized supernatant fraction of mixed cultures or with filter sterilized extracts formed by sonicating the pellet fraction (Table 3.1). Cultures amended with the supernatant fraction showed stimulation of dechlorination and the pellet fraction extract was even more stimulatory. A further transfer of the supernatant-amended culture

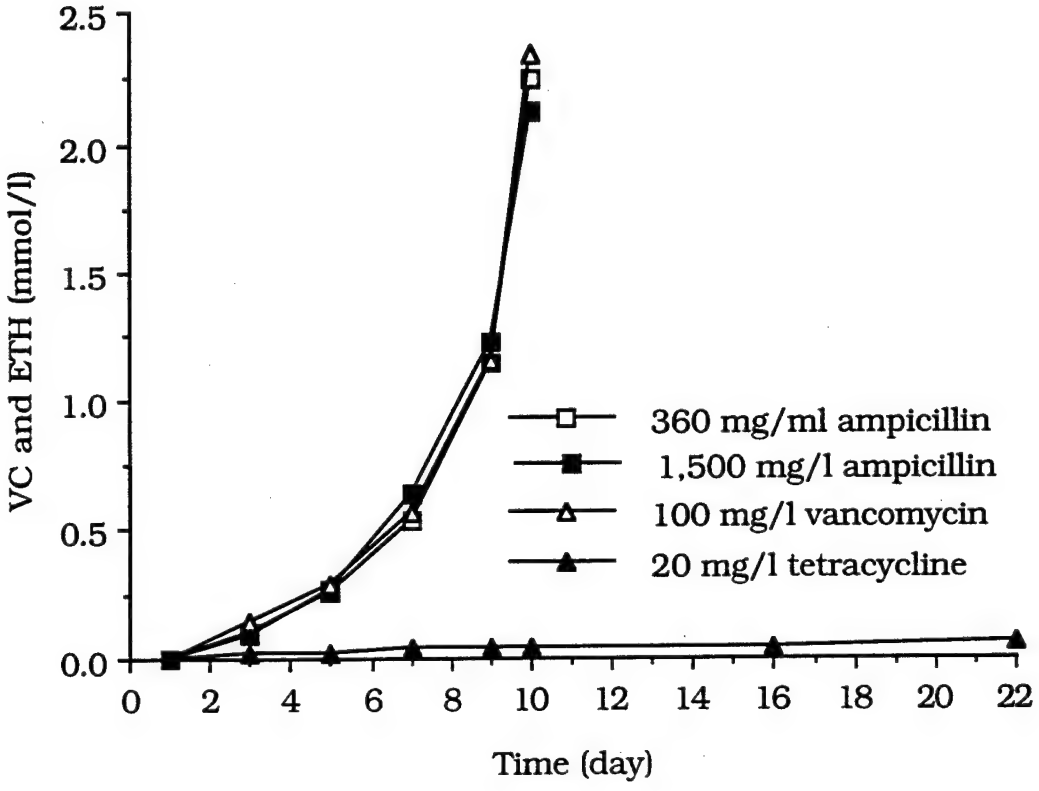


FIGURE 3.2 Product formation (accounted by VC and ETH) from PCE by cultures exposed for the first time to the antibiotics ampicillin, vancomycin and tetracycline.

TABLE 3.1 Effect of various nutrient amendments on product formation from PCE by cultures transferred a second time into ABSS-supplemented medium containing 0.3 g/l ampicillin. Values shown were taken after 23 days of incubation. Values less than 1 µmol/l are denoted as 0.

	Products (µmol/l)			
Additions	TCE	DCEs	VC	ETH
None	0	0	35	0
Mixed culture supernatant*	0	20	1,400	55
Mixed culture pellet extract**	0	37	2,130	172
Cholesterol (10 mg/l)	0	0	9	0
Horse Serum (15% v/v)		0	28	0
Volatile fatty acids	0	0	133	3
Yeast Extract (2 g/l)	203	83	288	0
Casamino acids (0.5 g/l)	0	30	1,600	70
Escherichia coli extract (15 % v/v)****	20	10	32	0
Clostridium pasteurianum extract (15% v/v)****	50	120	60	3

* Addition of 20% v/v supernatant of a mixed PCE dechlorinating culture incubated without ampicillin
** Extract from a cell pellet from a mixed culture equivalent to 10% v/v of the culture
*** Final concentrations: acetate, 29.7 mM; propionate, 8 mM; butyrate 3.2 mM, valerate, 0.92 mM; isovalerate, 0.91 mM; isobutyrate, 1.09 mM; 2 methyl butyrate, 0.82 mM
**** Total amount of cell biomass unknown, but much more than the culture pellet extract

into similar medium did not dechlorinate PCE, while the cultures amended with the pellet extract could be transferred indefinitely. These results indicate that one or more cell components of contaminating organisms were responsible for the stimulation.

Other potential nutrient amendments were also examined (Table 3.1). No stimulation of dechlorination occurred from addition of cholesterol and horse serum, two nutrients known to stimulate mycoplasmas, a group of eubacteria lacking cell walls. A mixture of volatile fatty acids (VFA) often used to stimulate the growth of certain anaerobes gave only slight stimulation of dechlorination, as did yeast extract. Casamino acids (Difco Laboratories, Detroit, MI) produced a notable stimulation, similar to that produced by the supernatant fraction of mixed cultures. Nevertheless, subsequent transfers of cultures amended with VFA, yeast extract or Casamino acids were not successful (data not presented). Significant stimulation was not detected from cell extracts of the Gram-negative aerobic eubacterium *Escherichia coli* or the Gram-positive anaerobic eubacterium *Clostridium pasteurianum* strain W5, indicating that the growth factor(s) is not ubiquitous in bacteria. Other amendments tested that are not shown in Table 3.1 and which did not sustain dechlorination were: Calf serum (6.85 mg/ml, CS); phosphatidyl choline (0.5 mg/ml, PC); Tween 80 (with albumin;, 0.1 mg/ml); LPS (0.25 mg/ml lipopolysaccharide) from *E. coli.* Also, the following next combinations of amendments did not sustain dechlorination: Tween 80 (with albumin)+PC; Tween 80 (with albumin)+VFA; cholesterol+LPS; YE+horse serum; YE+horse serum+Casamino acids+VFA.

Using a growth medium supplemented with filter-sterilized extract from mixed H_2/PCE cultures and ABSS(24), the PCE-dechlorinating organism (called Strain 195) was isolated by 10^7-fold dilution to liquid H_2/PCE medium containing 0.3 g/l ampicillin. These cultures, when transferred several times in the absence of ampicillin, showed no morphotypes other than irregular cocci detectable by phase-contrast and electron microscopy. No visible growth was detected in tests for contamination (sensitivity near 10 organisms/ml) using our basal growth medium amended with lactate, sulfate, and thiosulfate to detect sulfate reducers, amended with 0.2 g/l yeast extract to detect fermentative heterotrophs, or using Brewer's thioglycollate medium (Difco) to detect fermentative heterotrophs.

3.4.b Examination of requirements of Strain 195 for nutrients from mixed cultures.

Shortly after isolation, the extract from mixed H_2/PCE cultures was substituted by a more powerful extract derived from a culture which grew on butyrate as the e^- donor. This butyrate/PCE culture was derived from the original methanol/PCE culture (12) and was more diverse and had a higher cell density than the extract from the H_2/PCE culture (data not shown). As it can be seen in Fig. 3.3, both extracts (50x, 2.5% v/v) produced from enrichments grown on methanol or butyrate were able to sustain PCE dechlorination and could substitute for the extract from H_2/PCE cultures. In both cases, intermediate products (TCE and DCEs) were produced during each

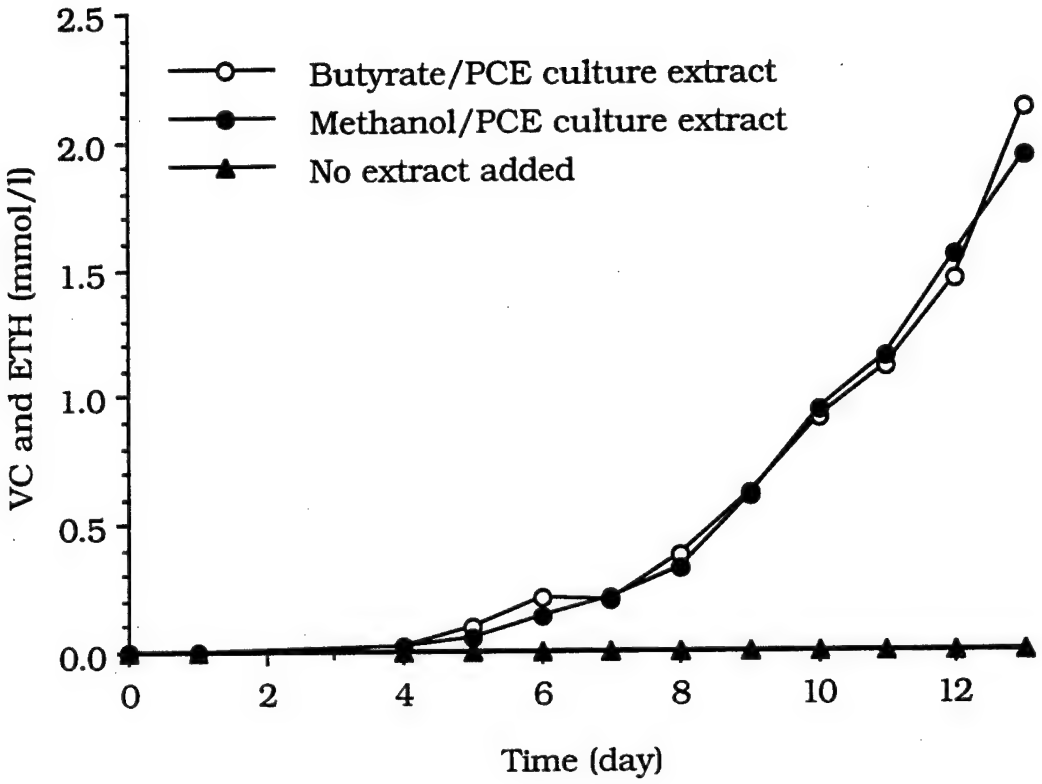


FIGURE 3.3 VC and ETH formation by cultures grown in the presence of ampicillin, amended with ABSS and either extract from a butyrate/PCE or a methanol/PCE culture, or not amended with any cell extract.

PCE feeding, which were dechlorinated to VC and ETH as PCE diminished (data not shown). Because of the availability of the butyrate/PCE culture, its extract was utilized as an amendment instead of the one from the methanol/PCE culture.

To test the optimal amount of butyrate/PCE culture extract required by Strain 195, several percentages (v/v) of 50x concentrated extract were amended to the culture (Fig. 3.4). The addition of 10% v/v butyrate/PCE culture extract produced the best PCE dechlorination by Strain 195. Cultures amended with less than 2.5% v/v dechlorinated PCE very slowly. Due to the difficulty of producing large amounts of the extract, it was decided to amend the cultures with 5% v/v butyrate/PCE culture extract from this point on. Six months after butyrate/PCE culture extract was used by the cultures for the first time, Strain 195's ability to dechlorinate had PCE increased considerably under the same conditions (Fig. 3.5). It should be noticed that the high amount of VC produced (up to 5.1 mmol/l VC) did not seem to affect the ability of the culture to dechlorinate PCE. The amount of stimulation of PCE dechlorination present in the fraction of the butyrate/PCE culture utilized so far (supernatant fraction of the extract; see Materials and Methods) was compared to the particulate fraction (pellet obtained after last centifugation) (Fig. 3.6). Over time, clearly the major part of the activity was present in the supernatant extract of the butyrate/PCE culture. Because French pressing the cells seemed to keep more of the activity in the supernatant fraction rather than in the particulate fraction, this method was chosen over sonication.

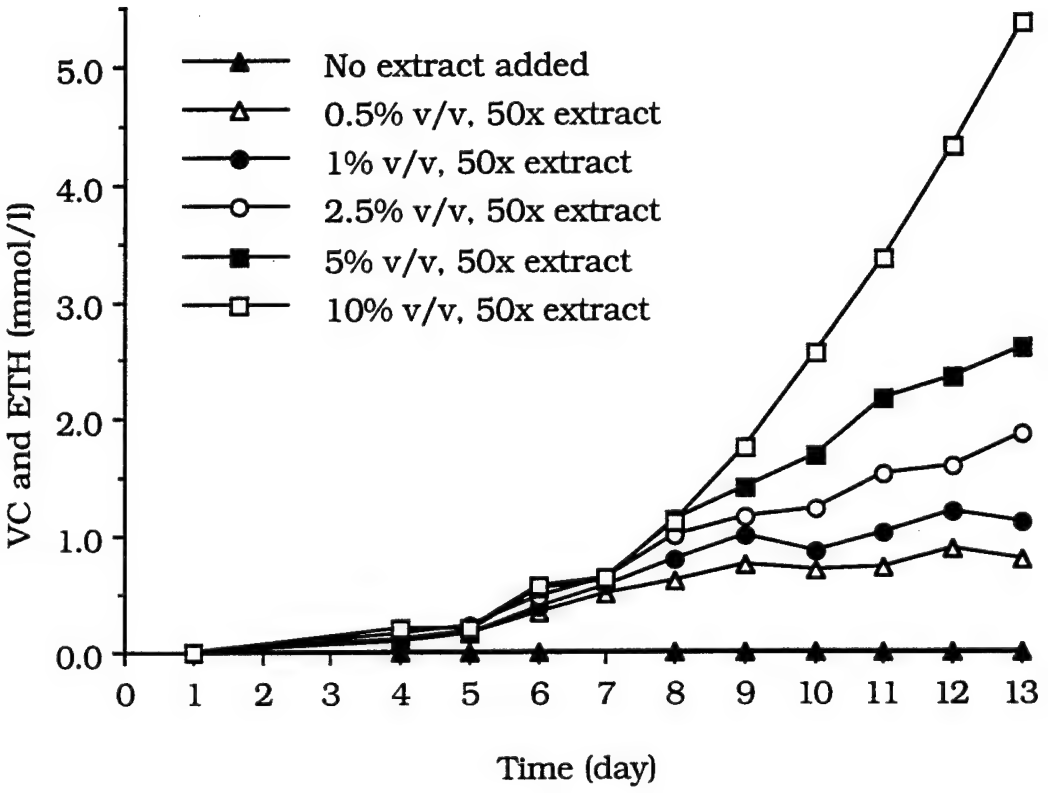


FIGURE 3.4 VC and ETH produced by Strain 195 in the presence of ampicillin and with different amounts of extract from the butyrate/PCE culture added.

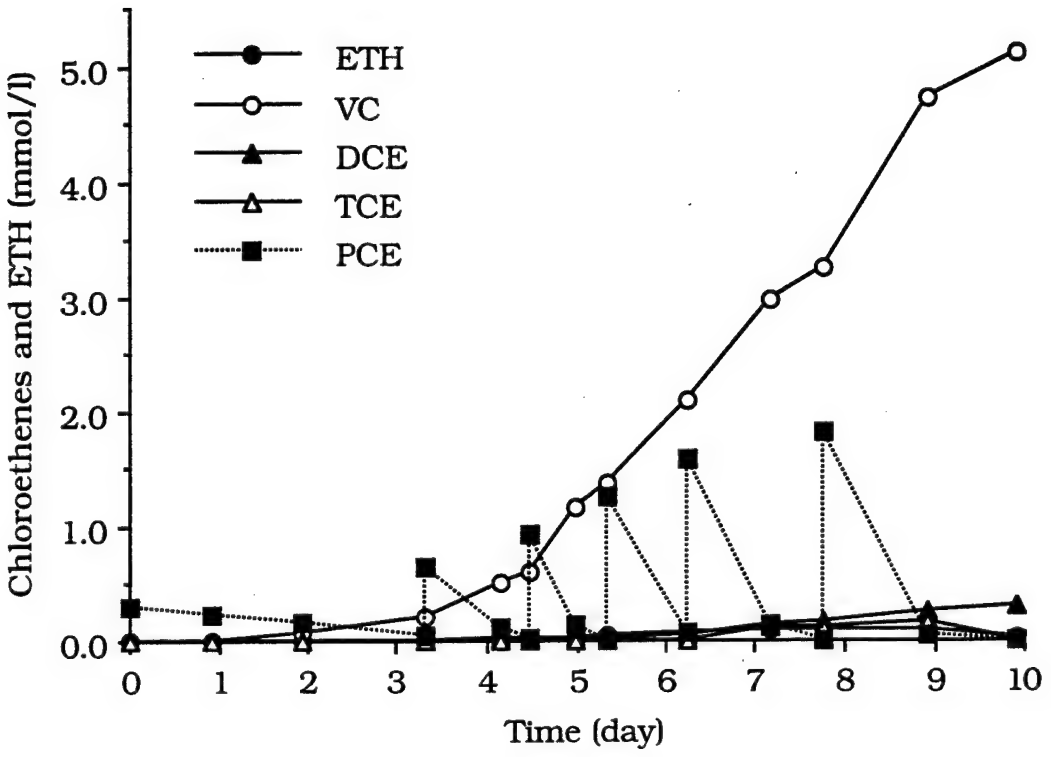


FIGURE 3.5 PCE degradation to VC and ETH by Strain 195 grown on ampicillin, ABSS, and 5% v/v, 50x Butyrate/PCE culture extract.

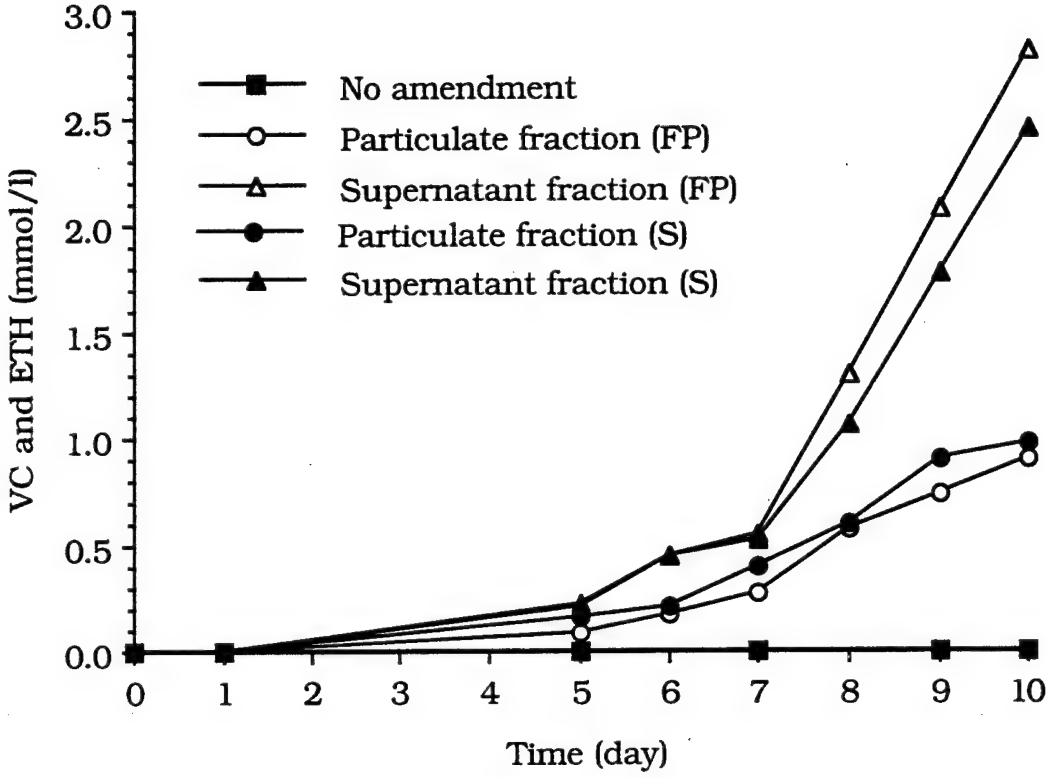


FIGURE 3.6 Product (VC and ETH) formation from PCE by Strain 195 grown with ABSS, 0.3 mg/ml ampicillin and 5% v/v of either the supernatant extract or the particulate fraction of a 50x concentrated Butyrate/PCE culture. FP = French pressed; S = sonicated.

To estimate the size of the molecule/s responsible for the activity of the butyrate/PCE culture extract (supernatant fraction), this extract was passed through 10,000 and 50,000 dalton Centricon filters and the filtrates and retentates were tested for their ability to support dechlorination in Strain 195. Results in Fig. 3.7 showed that the molecule/s responsible for the activity of the extract was greater than 50,000 dalton (the retentate kept most of the extract activity).

The butyrate/PCE culture extract was always kept frozen until utilized, and then it was stored at 4°C for about four weeks, when it was discarded and replaced by a fresh extract. During its time of use, the pH of the extract was 6.9.

3.4.c Growth and substrate utilization by Strain 195.

Fig. 3.8 shows growth of the culture on H_2 and PCE, as measured by increases in cell protein and direct microscopic cell counts, during metabolism of PCE to VC and ETH. Protein was quantified using the NanoOrange kit and cells were stained with acridine orange before being counted microscopically. There was growth of cultures until Day 5, and the estimated doubling time was 0.8 days. After Day 5, growth ceased while PCE dechlorination continued, suggesting uncoupling of growth from dechlorination. Cultures receiving H_2 but not PCE showed only slight initial growth (about 1 doubling), and PCE dechlorination products were not detected in uninoculated cultures. The amount of VC and ETH produced represented over 90% of the PCE added in inoculated cultures. The protein yield for Days 1-5 was 4.8 ± 0.3 g protein per

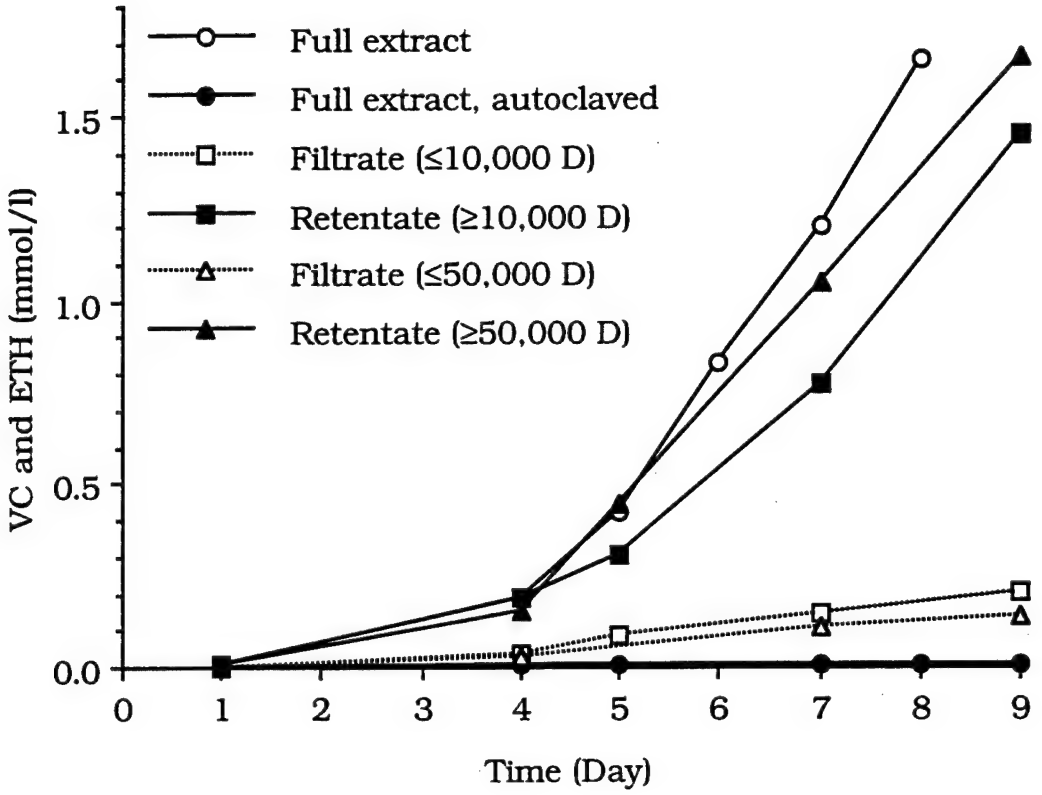


FIGURE 3.7 Effect of different fractions of the butyrate/PCE culture extract. The extract was filtered through 10,000 and 50,000 D diameter size Centricon filters at a centrifuging speed of 5500 rpm for 50 min. The ability to support PCE dechlorination by the filtrate and retentate were tested on Strain 195 grown with ABSS under standard conditions. Extracts were added at 5% v/v. A part of the full extract was autoclaved for 40 min at 121°C.

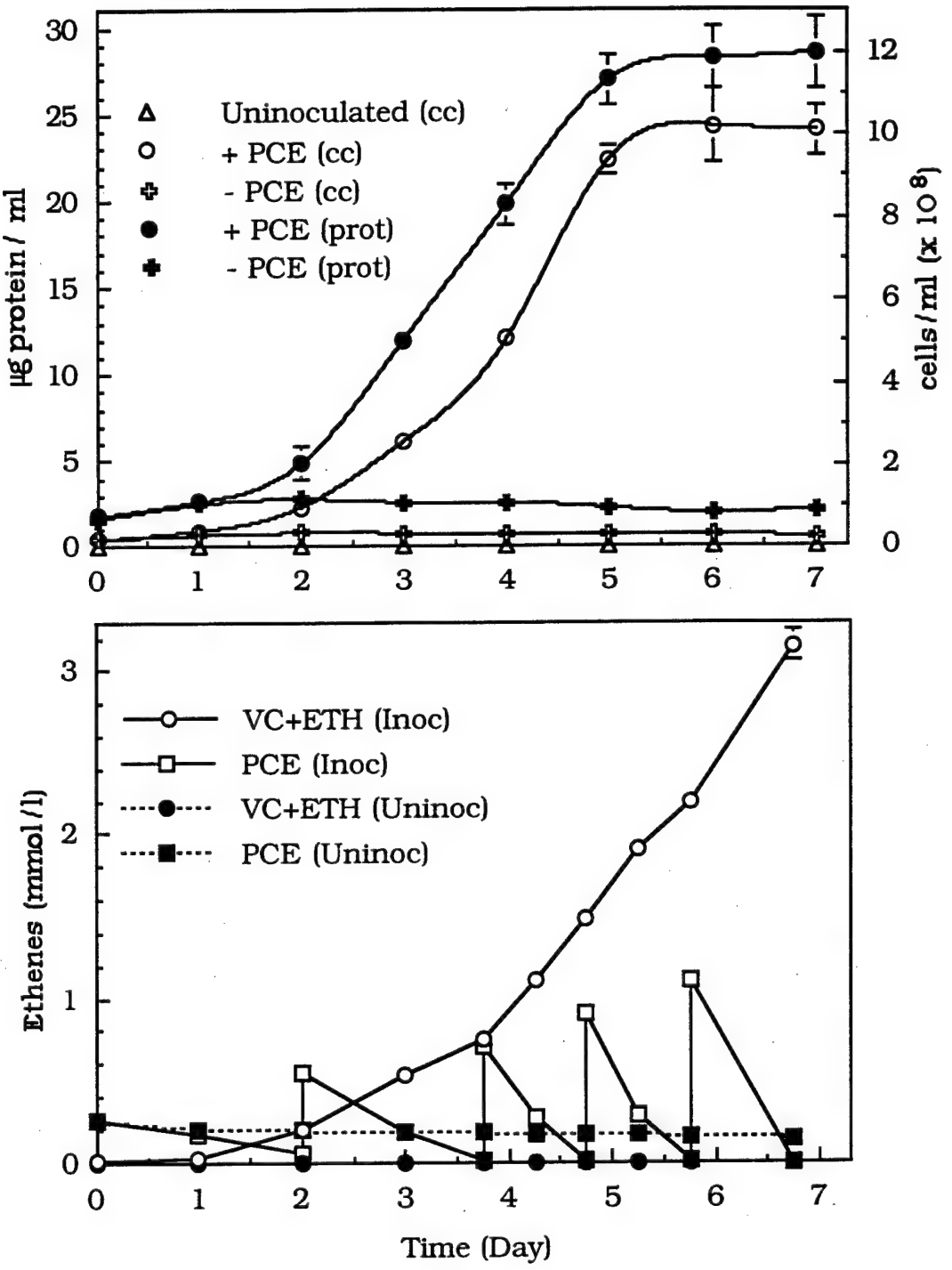


FIGURE 3.8 (a) Protein (µg/ml) growth curve and cell counts (number of cells/ml) for a pure culture of Strain 195 grown in medium with ABSS and 5% v/v butyrate/PCE culture extract. **(b)** Product formation from PCE dechlorination by the same culture, during the same experiment. Graphs show the means from triplicate tubes.

mol chloride released (determined from the dechlorination products formed), and a specific activity of 69.0 ± 10.5 nmol chloride released per minute per mg protein was determined. For comparison, *D. multivorans* has a growth yield of 1.6 g cell dry weight per mol chloride released, and a specific activity for whole cells of 150 nmol chloride released per minute per mg protein when dechlorinating PCE to *cis*-DCE (26).

Fig. 3.9 shows a time course for PCE conversion to ETH by Strain 195 by a culture which had received five previous doses of PCE amounting to 2.1 mmol/l. PCE was metabolized to VC at a nearly constant rate of 40 μmol per hour per liter of culture medium with little buildup of intermediates. VC dechlorination to ETH commenced after PCE depletion and could be well fit by first-order kinetics with a half-life near 80 hours before 300 hours had elapsed and one near 150 hours afterwards (regressions not presented), indicating a possible decay in the culture's ability to metabolize VC with time.

3.4.d Electron donor and acceptor utilization by Strain 195

We have performed a physiological characterization of the Strain 195, including examination of its ability to use e^- donors and acceptors commonly used by microorganisms carrying out anaerobic respiration (Table 3.2 presents a summary of nutritional experiments). H_2 was required for PCE reduction, and growth of the pure culture only occurred when both H_2 and PCE were present (Figs. 3.5 and 3.8). Among the potential e^- donors examined which did not support PCE dechlorination were methanol, pyruvate, lactate,

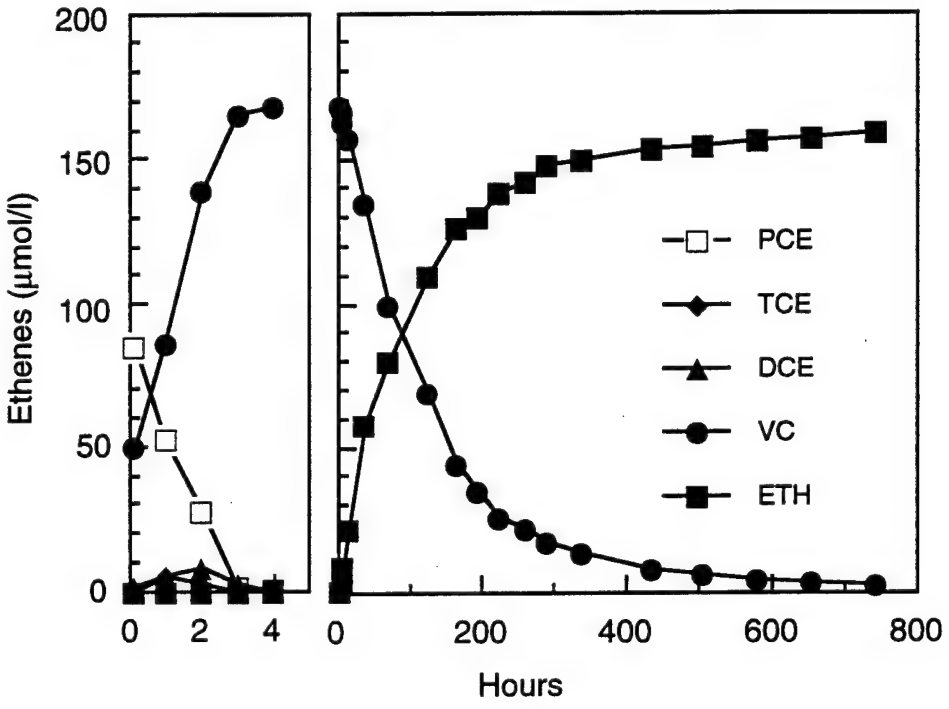


FIGURE 3.9 Conversion of ca. 130 µmol/l PCE to ETH by a pure culture of Strain 195. The culture was given several doses of PCE followed by flushing with N_2/CO_2, which removed all ethenes except for ca. 50 µmol/l VC. PCE was underestimated in early data points due to its slow dissolution into the aqueous phase (30).

TABLE 3.2 Substrates required, utilized and not utilized by Strain 195 (refer to the List of Abbreviations, in p. xv, for abbreviated names). §=experiment performed on Strain 195 when in the purified H_2/PCE culture; †=see Chapter 4.

	Requires	Uses	Does not use
Carbon sources	Acetate	YE	-
e^- donors	H_2	-	Formate Acetate§ Methanol Ethanol Pyruvate Glucose YE Lactate
e^- acceptors	PCE TCE *cis*-DCE 1,1-DCE DCA	DBA	*trans*-DCE VC Oxygen† Sulfate Sulfite Thiosulfate Nitrate Nitrite Fumarate
Others	Vitamin B_{12} SS Cell extract	CA YE Riboflavine(?) VFA(?)	Biotin§ Folic acid§ Pyridoxine HCl§ Thiamine HCl§ Nicotinic acid§ Ca Pantothenate§ p-aminobenzoate§ Lipoic acid§ Hemin§ Nicotinamide§ 1,4-Naphthoquinone§ *E. coli* extract *C. pasteurianum* extract Calf serum Horse serum LPS from *E. coli* Cholesterol Phosphatydyl choline Tween 80

ethanol, glucose, and yeast extract. None of these substrates supported growth when present alone in the absence of PCE. Surprisingly, formate was also not utilized by Strain 195 to support dechlorinationin the presence of 0.3 μg/ml ampicillin (Fig. 3.10). The combined results suggest that formate is not directly utilizable by Strain 195. In the mixed culture, a different microorganism was most probably responsible for the use of formate, which may have produced H_2 in the process, to be used by Strain 195 as the direct e^- donor for dechlorination.

Potential e^- acceptors which did not support growth when H_2 was provided as the e^- donor included sulfate, sulfite, thiosulfate, nitrate, nitrite, fumarate, and oxygen (see Fig. 4.3). The culture could reductively dechlorinate 1,2-dichloroethane and 1,2-dibromoethane to ethene, as did the original enrichment culture (30) (data not presented).

3.4.d Microscopic studies of Strain 195.

Electron microscopic examination of Strain 195 (Fig. 3.11) revealed non flagellated, pleomorphically shaped coccoid cells with sizes ranging from 0.3 to 0.9 μm in diameter. These cells had an unusual cell wall ultrastructure resembling neither typical Gram-positive nor Gram-negative peptidoglycan layers in eubacteria, but rather the S-layer protein subunit type cell walls found in many Archaea (21). To test for the presence of a peptidoglycan cell wall that could be located in the electrondense zone seen in between the cell membrane and the S-layer, we used fluorescently-labeled wheat germ

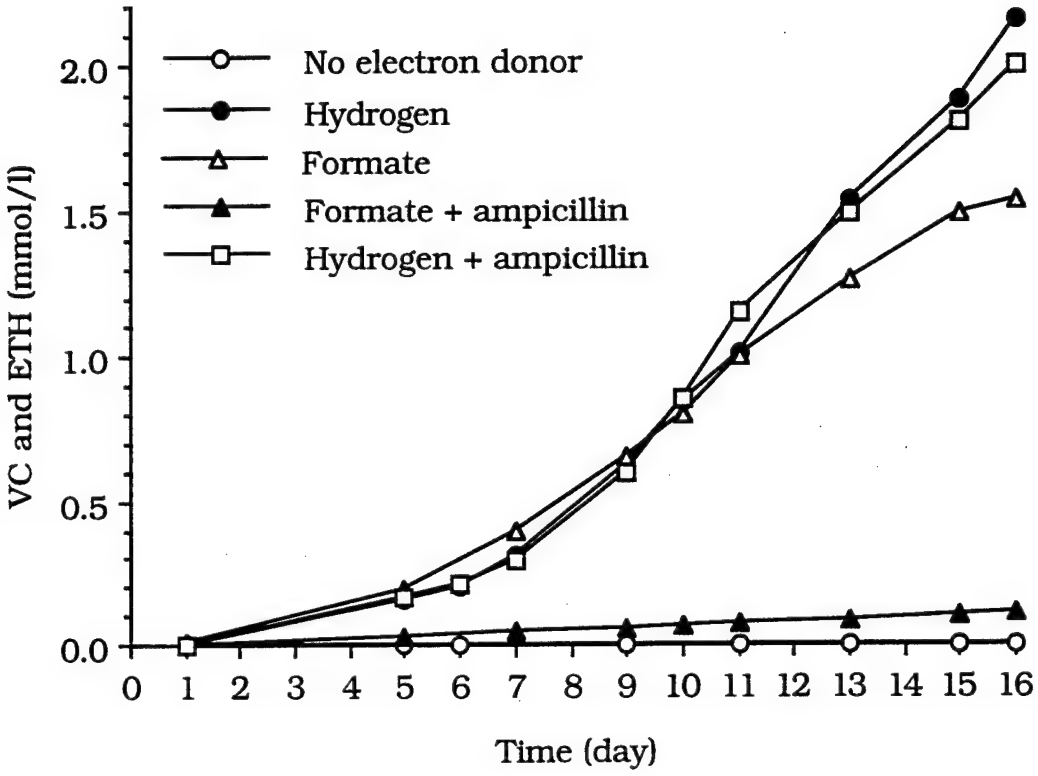


FIGURE 3.10 Formation of VC and ETH from PCE by Strain 195 with formate or hydrogen as electron donors and without any electron donor. Formate and hydrogen were also tested with and without 0.3 μg/ml ampicillin amended to the culture.

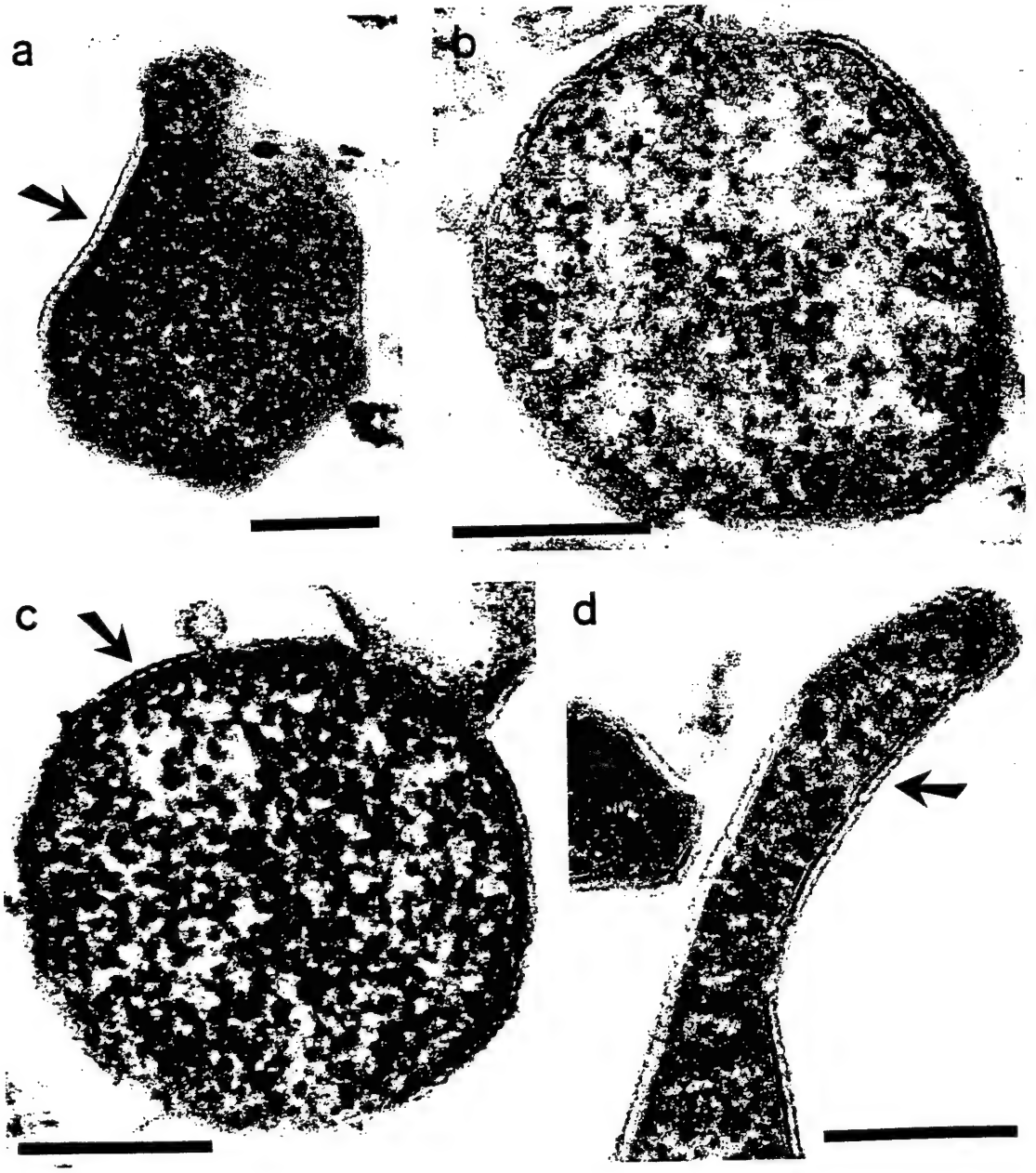
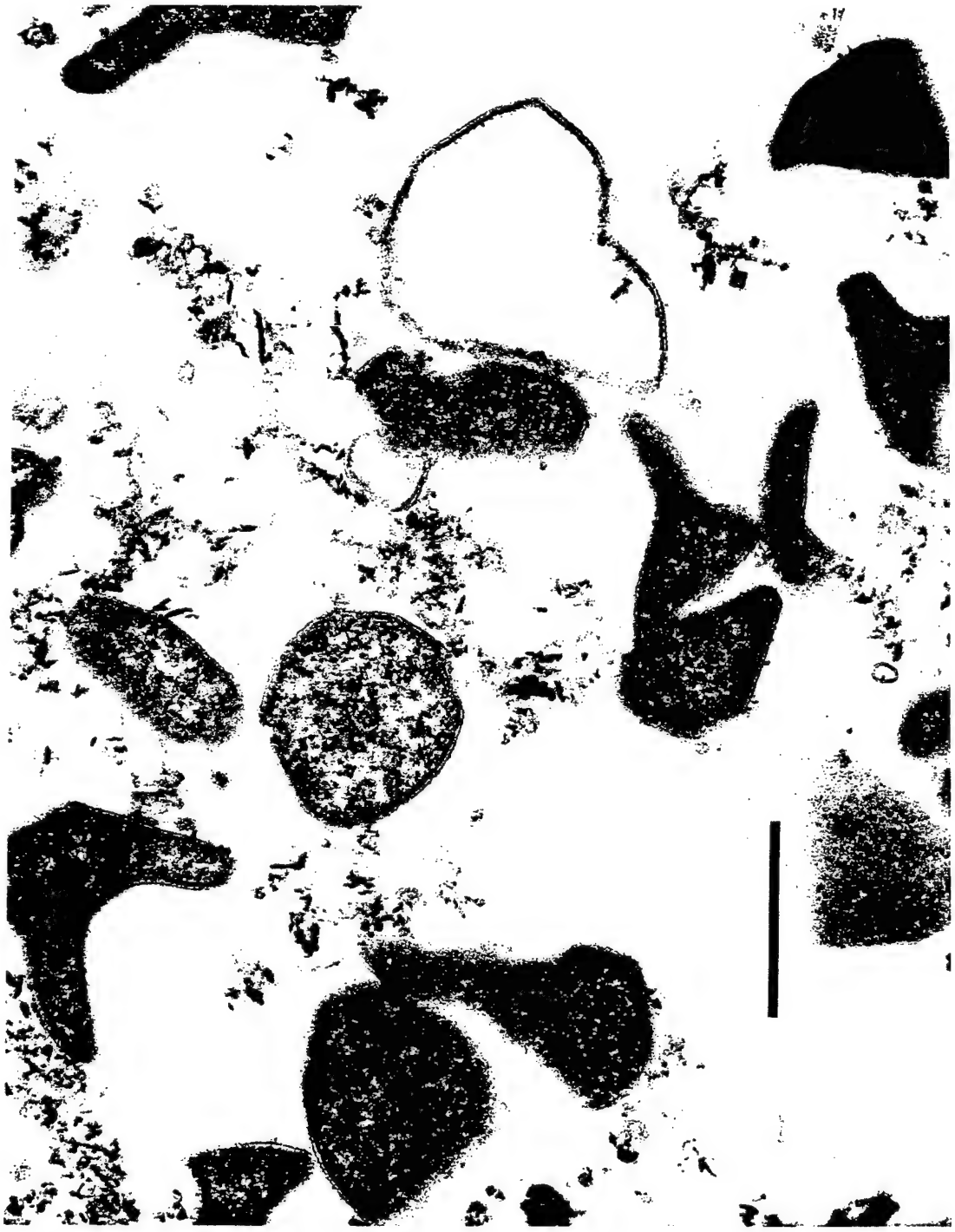


FIGURE 3.11 Thin-section uranyl acetate-stained electron micrographs of coccoid (**b**, and **c**) and flattened (**a** and **d**) cells of Strain 195 showing their characteristic S-layer type cell envelope signaled by arrows in **(a)**, **(c)** and **(d)**. Marker bars represent 0.2 μm. **(e)** General field of vision showing a central coocoid cell {seen in **(b)**}, several flatenned cells and an empty cell envelope. Marker bar represents 0.5 μm.

FIGURE 3.11 (Continued)



agglutinin, which specifically binds to N-acetylglucosamine and N-acetyl-neuraminic acid (28). This stain bound to whole cells of the Gram-positive eubacterium *Clostridium pasteurianum* WF (data not shown) and to cell-wall preparations of the Gram-negative eubacterium *Escherichia coli* DH5a (Fig. 3.12 c and d) {the outer membrane can protect the peptidoglycan from lectin binding in intact cells (28). No binding was detected for whole cells (not presented) or cell wall preparations (Fig. 3.12 a and b) of Strain 195.

3.4.e 16S rDNA analysis of Strain 195. (This experiment was performed by Y. T. Chien and analyzed by S. H. Zinder).

Because of its unusual antibiotic sensitivities and cell wall structure, it was of interest to determine the phylogenetic position of the Strain 195 based on its 16S ribosomal DNA sequence (Fig. 3.13). In this and all other phylogenetic analyses we performed, the PCE dechlorinator clearly grouped within the eubacteria, but did not cluster closely with any of the known phylogenetic lines. The analysis in Fig. 3.13 indicates that the PCE-dechlorinator is most closely related to a branch including cyanobacteria and planctomycetes, but no close affinity with either of these groups was found in more extensive analysis. DNA distance analyses placed the PCE-dechlorinator close to *Clostridium butyricum* and relatives (6), but there was little affinity for other members of the Gram-positive line. It has been found that the eubacterial branches are themselves robust, but they readily shift in branching order depending on the methods and representative species used in the phylogenetic analyses (32),

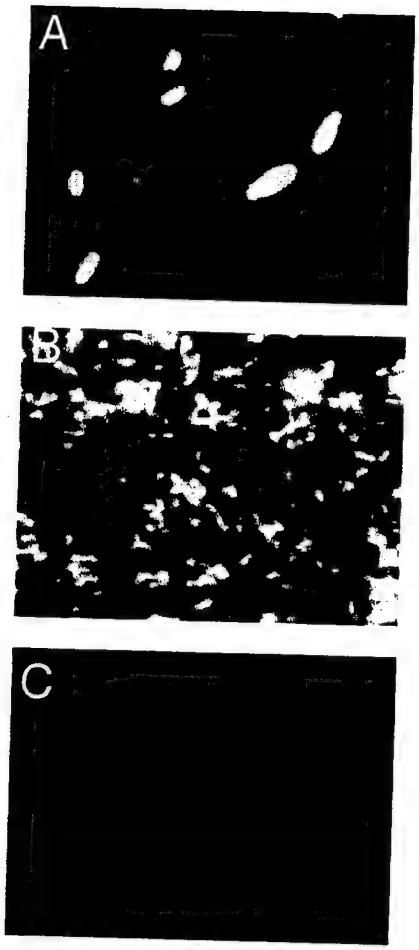


FIGURE 3.12 Epifluorescence micrographs of whole cells of *Clostridium pasteurianum* (**a**); cell wall fragments of *Escherichia coli* DH5a (**b**); and cell wall fragments of Strain 195 (**c**) stained with 100 mg/l fluorescein-labeled wheat germ agglutinin (28).

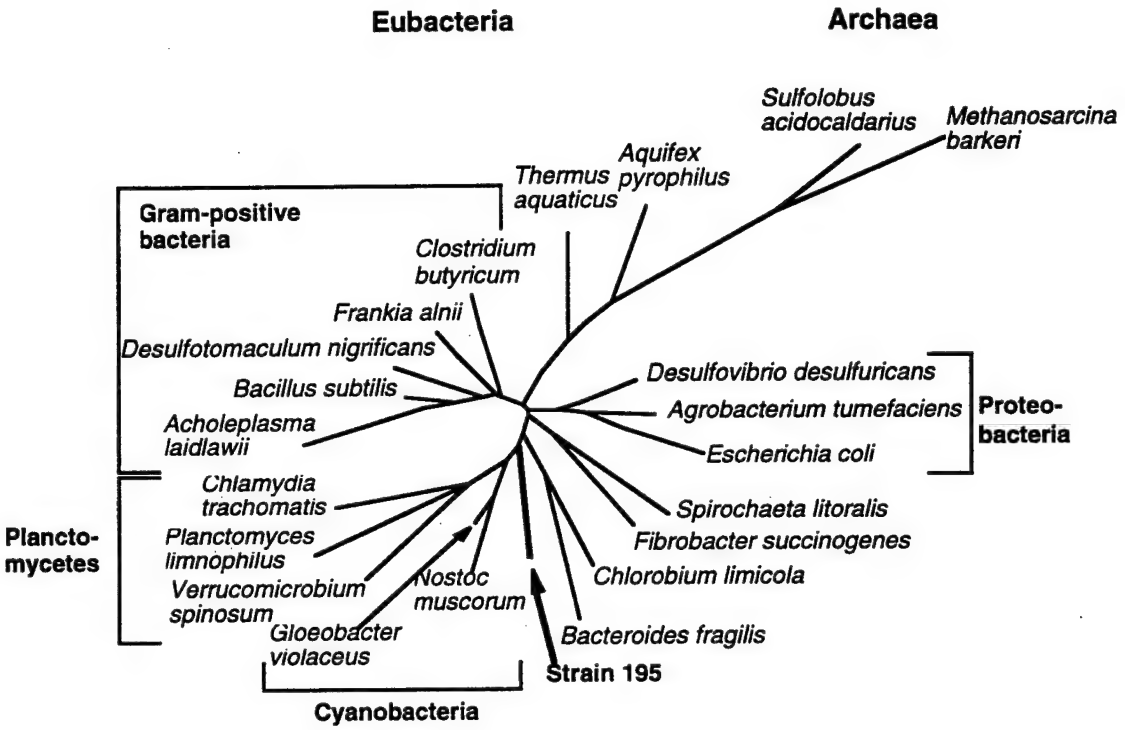


FIGURE 3.13. Unrooted phylogenetic tree generated for the 16S rDNA sequence from Strain 195 using the SUGGEST TREE maximum likelihood program provided by the Ribosome Database Project (RDP) (23). Some organisms included in the original analysis were deleted from the figure for simplicity. Other analyses of these sequences were performed by manually aligning Strain 195's sequence to other pre-aligned sequences from the RDP, followed by using the PHYLIP 3.5c package (11), including DNAML (maximum likelihood analysis), and DNADIST (Kimura model) coupled to either FITCH or NEIGHBOR. None of these analyses showed any clustering of Strain 195 within any known eubacterial group.

making the behavior of Strain 195 typical of a separate eubacterial branch. At this point, the most prudent taxonomic decision is to place the PCE-dechlorinator in a separate branch of the eubacteria. Because it does not apparently belong to any presently known genus or species, we have tentatively named Strain 195 "*Dehalococcoides ethenogenes*" but a formal taxonomic description must be made.

3.5 DISCUSSION.

A novel organism capable of reductive dechlorination of PCE to ETH, tentatively named *"Dehalococcoides ethenogenes"* strain 195, has been isolated. This is the first report of a pure culture that is able to completely dechlorinate PCE; all other organisms isolated so far replace the first two chlorines, leaving the other two untouched.

Isolation by dilution, although tried repeatedly, was not successful. PCE dehlorination in a dilution high enough to prevent the growth of heterotrophic contaminants was not attained. In roll tubes, the main factor was the smallness of the dechlorinating colonies. Production of chlorinated derivatives stopped before colonies responsible for the dechlorination process could be seen. This could be due to a requirement by "*D. ethenogenes*" strain 195 for nutrient(s) from a syntrophic organism. These nutrient(s) could easily be obtained by the dechlorinator in liquid medium, but in agar, diffusion would be impaired at higher dilutions due to the semisolid nature of agar and to the distance between cells separated by the dilution process. Also, the fact that PCE dechlorination leads to HCl

production may explain the smallness of the colonies. In solid medium, the pH in and around the colony could become so acidified that, after certain growth of the colony, it would prevent further growth. The higher TCE production seen in these cultures is similar to results in liquid culture, and it is probably an effect of the high solubility of TCE in HDC.

"D. ethenogenes" strain 195 seems to have a very limited number of substrates that it is able to process. Its dependence on factors from other organisms underscores the importance of nutritional interactions between the PCE-dechlorinator and other organisms in the culture. Such dependence is typical of anaerobes which tend to exist in interacting communities (35). The little use that this organism has for very rich substrates like horse or calf serum and its dependence on halogenated compounds generates questions about how specific the role of this organism in nature is. The results in Figure 3.9 resemble those for the mixed methanol/PCE culture from which it was derived (30) except for the relative rates of PCE and VC dechlorination. In the methanol/PCE culture, PCE was dechlorinated to VC at a rate of 12.5 μmol per hour per liter and VC was dechlorinated to ETH with a half-life near 17 hours. Had this pure culture performed similarity, the expected half life for VC should have been near six hours, more than tenfold shorter than was found. If *"Dehalococcoides ethenogenes"* strain 195 is responsible for VC dechlorination in mixed cultures, then some factor, perhaps nutritional, limits the rate of VC dechlorination in the pure culture. It is also possible that another organism or strain present in the

mixed culture is capable of more rapid VC metabolism.

The potential presence of an S-layer in an organism that lacks a peptidoglycan cell wall is interesting. As observed in Fig. 3.11, the cell envelope seems to contain an electron-dense periplasmic space in between the cell membrane and the S-layer. This could be a zone of important enzymatic activity for the cell, that may or may not be related to dechlorination activity. Fig. 3.11 (b) and (c) show what could be a membrane vesicle that extrudes from the cell envelope. These are sometimes formed when highly electropositive compounds come in contact with cells, displacing Ca^{2+} and Mg^{2+} from the membrane (3).

Our knowledge of the diversity of organisms capable of respiratory reductive dechlorination is increasing (25). Once there is greater understanding of the diversity and properties of these organisms, questions can be addressed concerning their evolution and population biology. An interesting question regards the origin of organisms capable of attacking halogenated organic compounds, most of which have only been extensively released into the environment for less than 100 years, although some haloorganics are produced biogenically and abiogenically (17). Clues about the origin of these organisms may be obtained by examining their phylogenetic positions. *Dehalobacter restrictus* and *Desulfitobacterium* sp. strain PCE1 cluster with Gram-positive sulfate-reducing bacteria (represented in Fig. 5 by *Desulfotomaculum nigrificans*) while *Dehalospirillum multivorans* clusters with Gram-negative sulfate-reducing bacteria and campylobacters (represented by *Desulfovibrio desulfuricans*). Both of these groups tend

to be versatile at using respiratory electron acceptors including other chlorinated organic compounds such as chlorinated benzoates and phenols (5, 7, 31). The PCE-dechlorinating organism described here, however is divergent from these groups.

Another interesting question is whether these dechlorinating organisms evolved independently in different contaminated sites, or whether only a limited number of organisms have evolved this ability and need to be transported from other sites. These types of questions are not strictly academic. For example, at some contaminated sites, PCE is hardly dechlorinated at all, while at others it is only dechlorinated as far as *cis*-DCE, reminiscent of reactions carried out by *Dehalobacter restrictus* and *Dehalospirillummultivorans*, while at still other sites dechlorination proceeds to VC, ethene, or occasionally ethane (18). It is not clear whether incomplete dechlorination at a given site is due to suboptimal physiochemical conditions, or to deficiencies in electron donors or nutrients present, or to the lack of appropriate organisms. Such knowledge will be useful in predicting fates of chloroethene contaminants, as well as possibly providing strategies for hastening their degradation.

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CHAPTER FOUR

FURTHER CHARACTERIZATION OF "*Dehalococcoides ethenogenes*" strain 195

The microbe is so very small
You cannot make him out at all"

Hilaire Belloc, 1870-1953
More beasts for worse children (1897), 'The microbe'

4.1 ABSTRACT.

"D. ethenogenes" strain 195 was further characterized. This organism had an optimum dechlorinating temperature of 35°C. It could reductively dechlorinate PCE at about 15°C, but with much slower dechlorination rates, and it did not dechlorinate PCE when incubated at 10°C. Its optimal pH was from 6.8 to 7.5, although good dechlorinating activity was obtained at pH 7.9. No PCE dechlorination was observed at pH 5.8. *"D. ethenogenes"* was unable to dechlorinate in the presence of oxygen and, after oxygen was removed, the culture seemed to be permanently inhibited by this compound. The culture dechlorinated faster at osmolalitites two to three times higher (200 to 300 mOsm) than that in the standard medium (80 to 110 mOsm). Platinum-replica studies of the cell envelope indicated that *"D. ethenogenes"* strain 195 possessed a highly structured S-layer with hexagonal symmetry and a separation of 15 nm between the center of any two of its contiguous globular subunits.

4.2 INTRODUCTION.

Because of the uniqueness of *"D. ethenogenes"* strain 195, due to its capabilities as a PCE dechlorinator and to its unusual 16S rDNA derived phylogeny and antibiotic sensitivities (9, 10), it was of interest to characterize this organism further.

From an environmental point of view, there are many parameters to consider that will influence a general degradation process if *in situ* or on site bioremediation is to be achieved eventually with a specific microorganism (1). Some of the studies performed on *"D. ethenogenes"* strain 195 in the last chapter have solely dealt with the nutritional requirements which allow this organism to grow with the fastest rates of dechlorination possible under the present circumstances. Nevertheless, the necessity to know as much as possible not only about the dechlorination process itself but also about the organisms that carry it out, make imperative further characterization of *"D. ethenogenes"* strain 195. Factors like optimal temperature, pH, reducing conditions and osmolality of the medium are important to improve the dechlorination process in the laboratory, but, most importantly, ultimately this information may be valuable to the detoxification of a particular contaminated environment.

Some of these studies have been performed on the isolates that are capable of reducing PCE to *cis*-DCE (3, 7, 8, 11, 14). Optimal temperatures vary from 21 to 38°C (from 21 to 31°C for Strain TT4B; from 34 to 38°C for *Desulfitobacterium* sp. strain PCE1; 37°C for *Enterobacter* sp. strain MS-1; from 25 to 37°C for *Dehalospirillum*

multivorans; and from 25 to 35°C for *Dehalobacter restrictus*). Optimal pHs vary from 6.8 to 7.8 (7.4 for Strain TT4B; 7.8 for *Desulfitobacterium* sp. strain PCE1; 7.0 for *Enterobacter* sp. strain MS-1; from 7.0 and 7.5 for *D. multivorans*; and from 6.8 and 7.6 for *D. restrictus*).

A deeper characterization of *"D. ethenogenes"* strain 195's S-layer ultrastructure is of interest because it has not been recognized so far in any of the other PCE dechlorinating isolates (7, 8, 13, 14).

4.3 MATERIALS AND METHODS.

4.3.a Chemicals and analyses of chloroethenes.

PCE and its chlorinated derivatives, ETH, H_2, and all other chemicals were purchased and utilized as described in Chapter Two.

Quantitative analysis of chloroethenes and ETH was performed as described in Chapter Three. Peak areas were calculated using the software supplied with the GC, and were compared to standard curves for chloroethenes (4). Headspace samples were 100-µl in all cases.

4.3.b Growth medium and culture conditions.

The ingredients utilized to produce the basal medium and the protocol followed to produce it, previous to the addition of the amendments, was followed as described in Chapter Two of this dissertation. The basal medium received the same sterile and anaerobic additions described in Chapter Three, including ampicillin.

The protocol for the preparation of the cell extract from the butyrate/PCE culture was also as described in Chapter Three. Inoculum sizes were 2% v/v, all incubations were done in duplicate, and each experiment presented was performed at least twice with similar results. Duplicate tubes performed similarly in the experiments presented (less than 5% difference in results for tubes under same conditions), so the results for some of these experiments are presented for individual tubes. Cultures were incubated as described in Chapter Three. H_2 and $NaHCO_3$ were added during the experiments as described in Chapter Two.

Temperature and pH optimum were obtained by following the increase in product formation (VC and ETH) by cultures incubated at different temperatures and pHs. Doubling times were obtained from semilogarithmic plots of product formation (r^2 values> 0.90), obtained during the exponential phase of growth of the culture. Values of $\mu_{max(VC+ETH)}$ were obtained from the doubling times. The pH was adjusted to the different values with 0.5 M HCl or 0.5 M NaOH before autoclaving and the experiment was performed at 35°C. For the pH experiment, product formation was measured only during the first 4 days and the pH of each tube was measured after the conclusion of the experiment to confirm the initial pH value (VARIANCE for duplicate tubes were not higher than 0.2 units of pH).

For the study of oxygen tolerance, oxygen was added at 21% by flushing the tubes with 30%N_2/70%CO_2 to purge the Na_2S. A mild vacuum was then produced in the tubes and air was let in through a venting needle. After tubes became pink (aerobiosis as indicated by

0.05 g/l resazurin present in the medium), H_2 and all amendments for growth, as well as the buffer system, were replenished. To re-reduce the medium, tubes were purged with 30%N_2/70%CO_2 and further reduced with Na_2S, as described in Chapter Two of this dissertation.

For the osmolality studies, five sets of duplicate tubes were prepared. The osmolarity of the medium was changed with a 1M solution of sucrose. The first set of tubes had no sucrose addition; the second had a final sucrose concentration of 0.05 M; the third of 0.1 M; the fourth of 0.2 M; and the fifth of 0.35 M. The osmolality of the solutions was measured with a Micro-osmometer, Model 3MO plus (Advanced Instruments, Inc., Norwood, MA).

4.3.c Microscopy.

A Zeiss Standard 18 microscope was used for phase-contrast observation of cells. For replica studies of *"D. ethenogenes"* strain 195, cultures were sampled at the end of their growth phases. A high vacuum freeze-etching unit BAF 400 K (Baltec, now Technotrade International, Manchester, NH) was used to produce the replicas. Two types of runs were performed. For the room temperature run, four freshly cleaved pieces of mica were placed on the specimen table. A drop of *"D. ethenogenes"* strain 195 cells in normal culture medium was placed on each of two of the pieces of mica and a drop of 40X concentrated culture was placed on each of the other two pieces. The specimen table with the samples was inserted into the chamber were the samples were coated with 4 to 5 nm of platinum. Samples where then stabilized with a carbon evaporation and removed from the

chamber. Replicas were separated from the mica onto the surface of distilled water, inside a glass well. Concentrated sodium hypochlorite (bleach) was gradually added and water gradually removed until the replicas were floating in full strength bleach. After 4 hours in bleach, the replicas were picked up in formvar-coated grids and viewed on a Philips EM201 electron microscope.

For the cold temperature run, the procedure followed was the same as for the room temperature run, but before coating, the samples were cooled to -130°C for 30 min. After coating the samples with platinum and carbon, they were removed from the chamber and allowed to warm to room temperature.

4.4 RESULTS.

4.4.a Optimal temperature and pH analyses.

Fig. 4.1 depicts $\mu_{(VC+ETH)}$ at different temperatures. Variances for all points are plotted (the ones not shown are smaller than the symbols). The temperature that allowed the fastest PCE dechlorination was around 35°C. The culture did not tolerate temperatures above 40°C. Below the optimal temperature, as the temperatures lowered, so did the speed of dechlorination and the doubling times, being very low at around 15°C (doubling time of 16 days), although above zero.

Because *"D. ethenogenes"* strain 195 had been growing at 35°C for many years, it was possible that the culture needed one or two

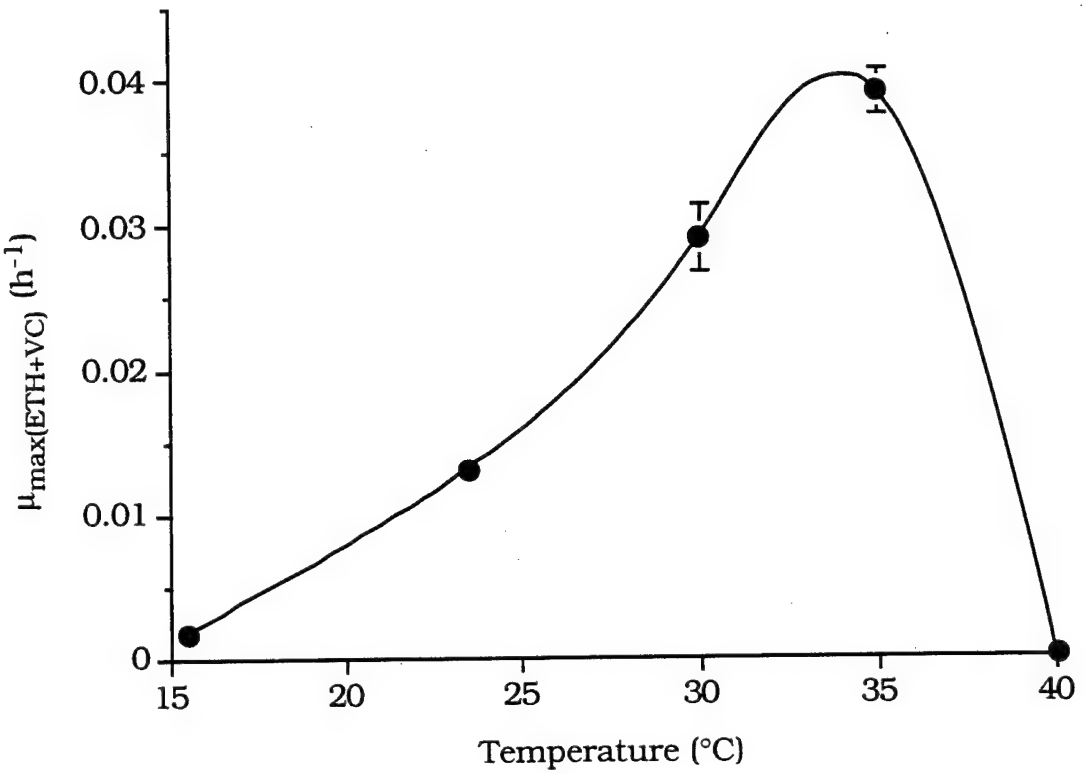


FIGURE 4.1 Determination of optimal temperature for PCE dechlorination by *"D. ethenogenes"* strain 195. μ values were obtained from doubling times, which were calculated from semilogarithmic plots of ETH and VC formation by cultures incubated under different temperatures.

more transfers to become accustomed to lower temperatures. To determine this, at Day 38 in the experiment of Fig. 4.1, one of the cultures incubated at 15.5°C was transferred into fresh medium with all the requirements for growth. Two of the newly transferred tubes were incubated again at 15.5°C while two others were incubated at 10°C (data not shown). After 31 days, the cultures incubated at 15.5°C had a maximum $\mu_{(VC+ETH)}$ of 0.0031 h^{-1} (equivalent to a doubling time of 9.3 days), almost twice as fast as the first generation cultures. After 76 days, the cultures incubated at 10°C had not produced any VC or ETH. At Day 31, one of the 2nd generation cultures incubated at 15.5°C was transferred into fresh medium with all the requirements for growth, and incubated again at 15.5°C (data not shown). After 25 days, the cultures had a maximum $\mu_{(VC+ETH)}$ of 0.00295 h^{-1} (equivalent to a doubling time of 9.7 days), very similar to the one detected for the second generation cultures.

Fig. 4.2 depicts $\mu_{(VC+ETH)}$ at different pHs. The optimal pH for PCE dechlorination by "*D. ethenogenes*" strain 195 incubated at 35°C comprised an interval between 6.8 and 7.5. Lower dechlorinating activity was obtained at pH 6.3 and 7.9. Cultures incubated at pH 7.9 started dechlorinating later (two days) than the rest (except the ones at pH 5.8), and the doubling times for these cultures were obtained between days 3 and 5 (for the other cultures they were obtained between days 2 and 4). No PCE dechlorination was observed at pH 5.8. For both the pH and the temperature analyses, PCE was never allowed to reach zero.

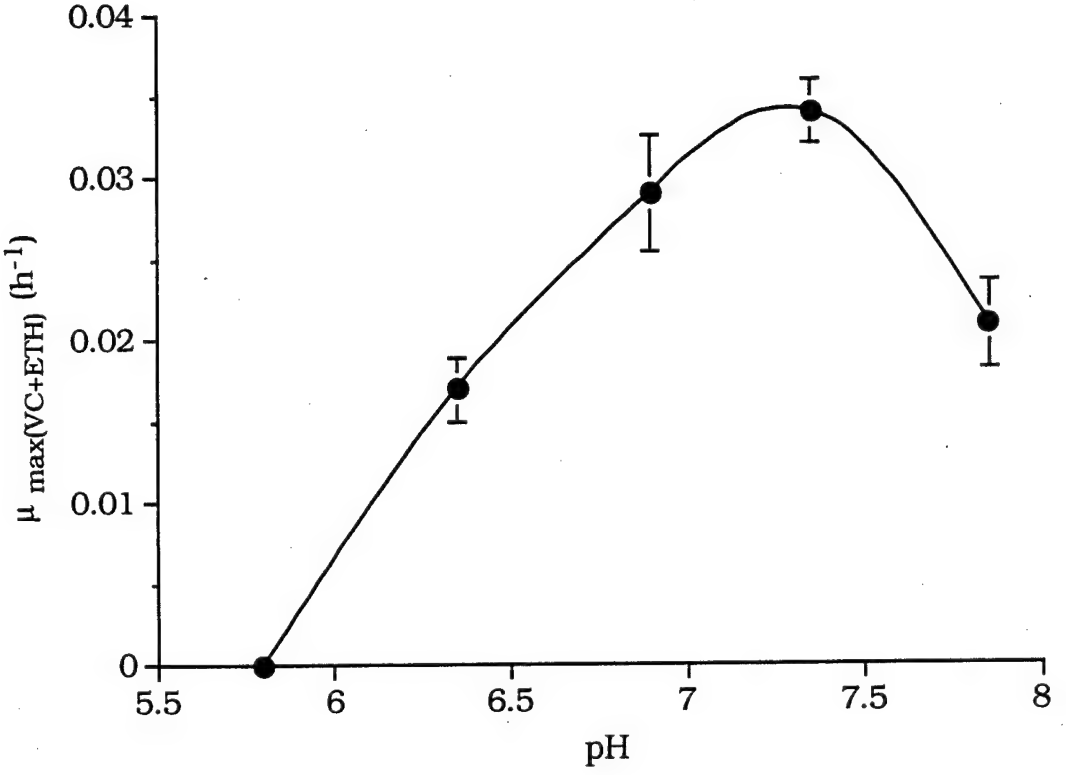


FIGURE 4.2 Determination of optimal pH for PCE dechlorination by *"D. ethenogenes"* strain 195. μ values were obtained from doubling times, which were calculated from semilogarithmic plots of ETH and VC formation by cultures incubated under different pHs.

4.4.b Effects of oxygen on PCE dechlorination.

To determine the ability of *"D. ethenogenes"* strain 195 cells to resist oxygenic conditions, a culture was transferred into fresh medium and given three consecutive doses of PCE. The culture was oxygenated at Day 7 at normal air concentrations (about 21%) (Fig. 4.3). Oxygen inhibited reductive dechlorination of PCE by the culture for the next six days. At Day 13, the cultures were reduced again. After this re-reduction, *"D. ethenogenes"* strain 195 did not dechlorinate PCE for 15 days, when the experiment was terminated. A similar experiment performed with a concentration of oxygen of 2% showed similar results (data not shown).

4.4.c Osmolality studies.

A study was pursued to determine how *"D. ethenogenes"* strain 195 coped with water activity (osmolality) in the medium and how did it compare to other organisms which also lack a peptidoglycan cell wall, like the mycoplasmas (18).

The osmolality of our basic salts medium was 27 mOsm (variance=0) and that of the sludge supernatant (SS), which is added at a 25% v/v, was of 129.5 mOsm (variance=3). When the complete medium in which cultures are transferred was measured, its osmolality was 82.6 mOsm (variance=3.5). The osmolality of a grown culture was around 110 mOsm (variance=6), higher than that of the same medium when just inoculated.

Fig. 4.4 shows the product formation from PCE dechlorination by several cultures incubated under different osmolalities. The inocu-

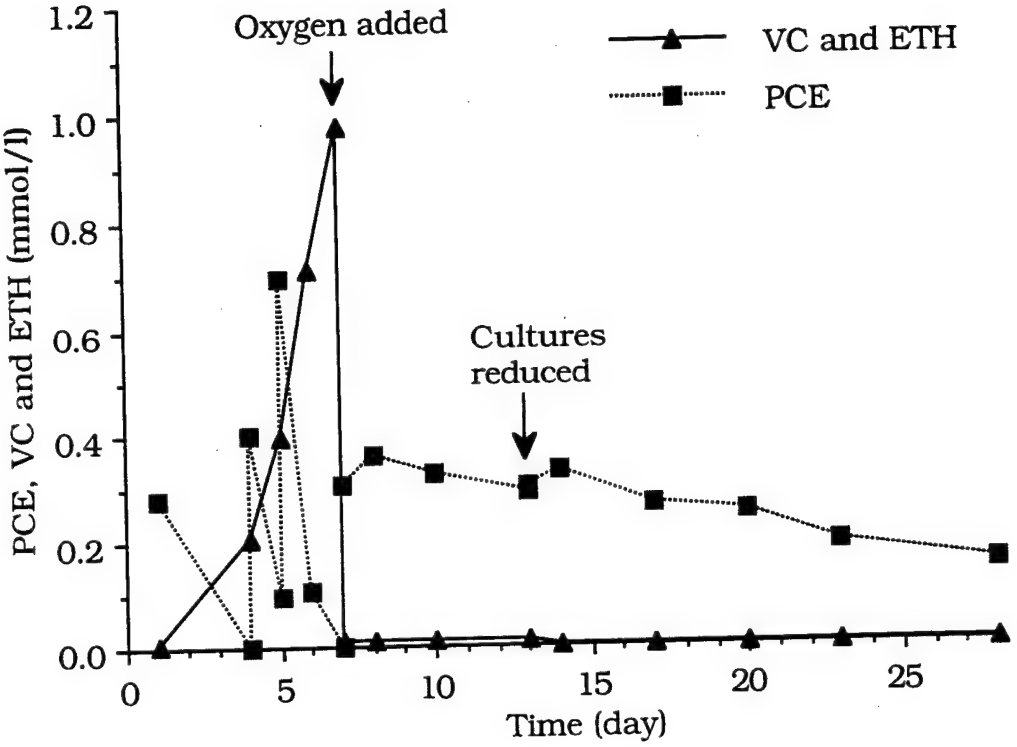


FIGURE 4.3 Product formation from PCE. The new transfers were fed three consecutive doses of PCE (note separate Y axis for PCE) and, at Day 7, oxygen (21%) was added. The cultures were re-reduced on Day 13.

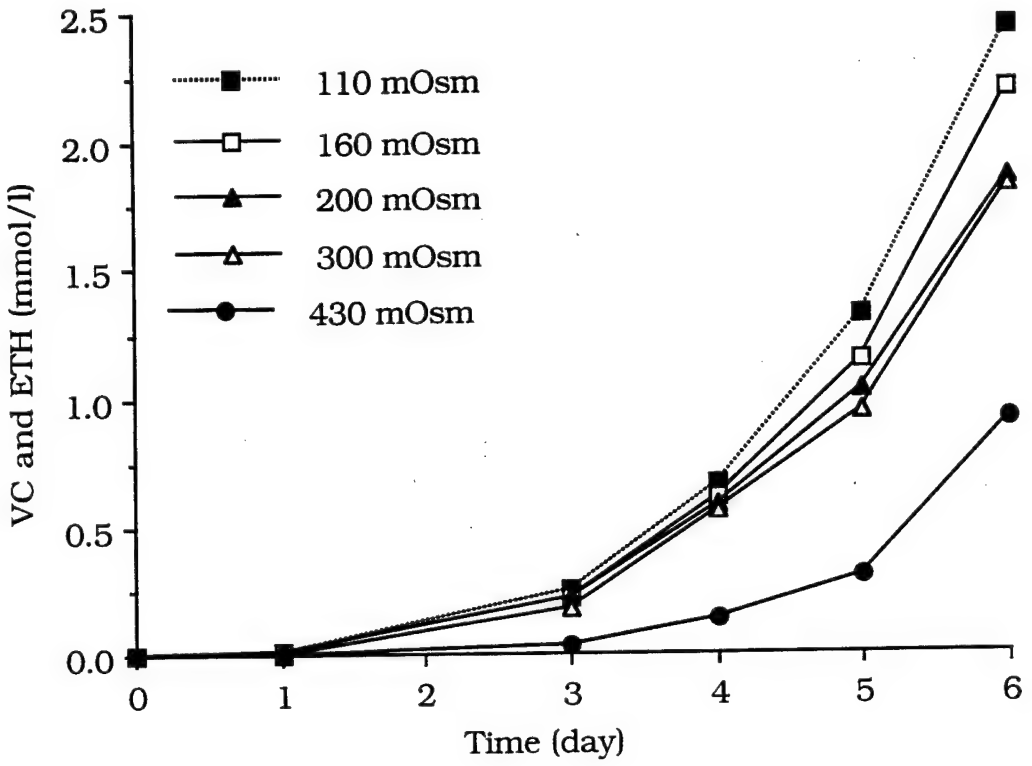


FIGURE 4.4 VC and ETH formation from PCE in cultures that grew and dechlorinated in media of different osmolalities.

lum utilized for all cultures came from a culture grown at the usual osmolality (80 to 110 mOsm). "*D. ethenogenes*" strain 195 growing in medium at 110 mOsm showed the best dechlorination rates. It is clear that the closer the osmolality of the medium to the normally utilized one, the better the dechlorination of PCE. Medium at 430 mOsm also sustained dechlorination but much poorly than the rest of the cultures.

The cultures growing at 110, 160, 200, and 300 mOsm were transferred again under the same conditions. The results in product formation from PCE are shown in Fig. 4.5. In this experiment, the order in which cultures at different osmolalities dechlorinated PCE, from best to worse, inverted itself with respect to the results of Fig. 4.4 (cultures at 430 mOsm were not tested in Fig. 4.5). Cultures at 200 and 300 mOsm performed better than those at 110 mOsm.

4.4.d S-layer studies.

To determine if the envelope structure seen in the micrographs of Figs. 3.11 contained indeed an S-layer, platinum and carbon coated replicas of "*D. ethenogenes*" strain 195 were produced and observed under electron microscopy. Fig. 4.6 shows six of the photomicrographs obtained from a pure culture of "*D. ethenogenes*" strain 195. The coccoid cells observed did not seem to have a rigid cell envelope. They were quite pleomorphic and many cells were folded onto themselves. The two-dimensional, crystalline S-layer lattice symmetry seemed hexagonal (15, 17) and it had a separation of 15 nm between the center of any two of its contiguous globular subunits. As

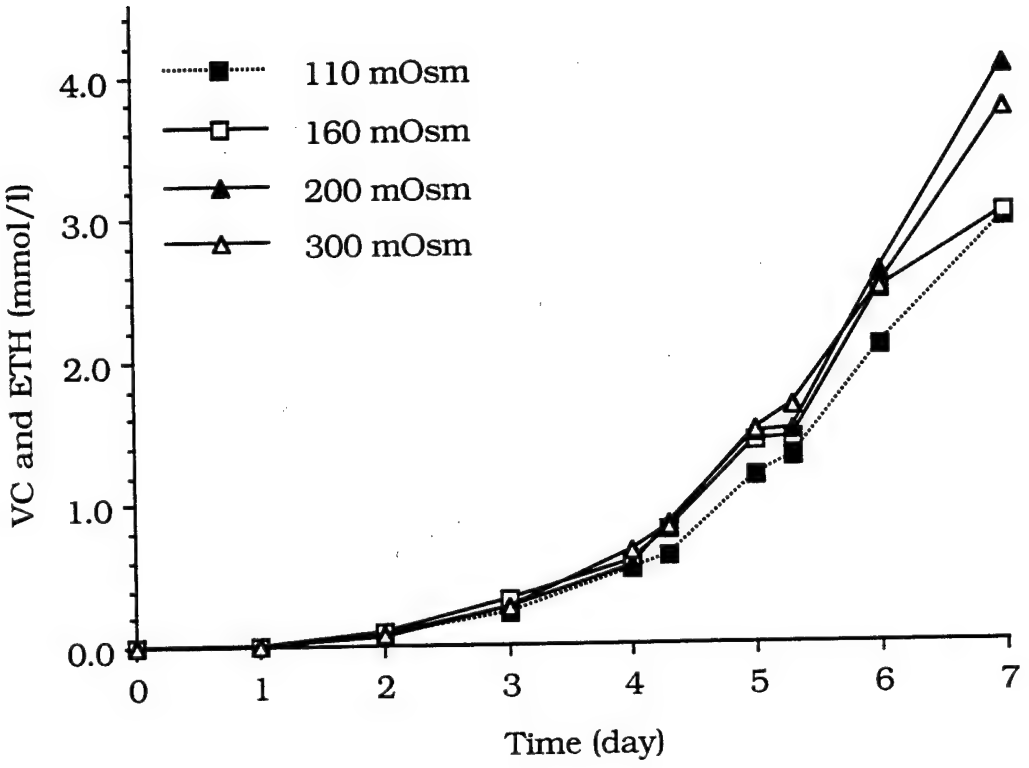


FIGURE 4.5 Product formation (VC and ETH) in cultures growing and dechlorinating in media of different osmolalities. The inoculum for each of the osmolalities represented in this graph was obtained from each of the cultures shown in Fig. 4.4 that had the same osmolality.

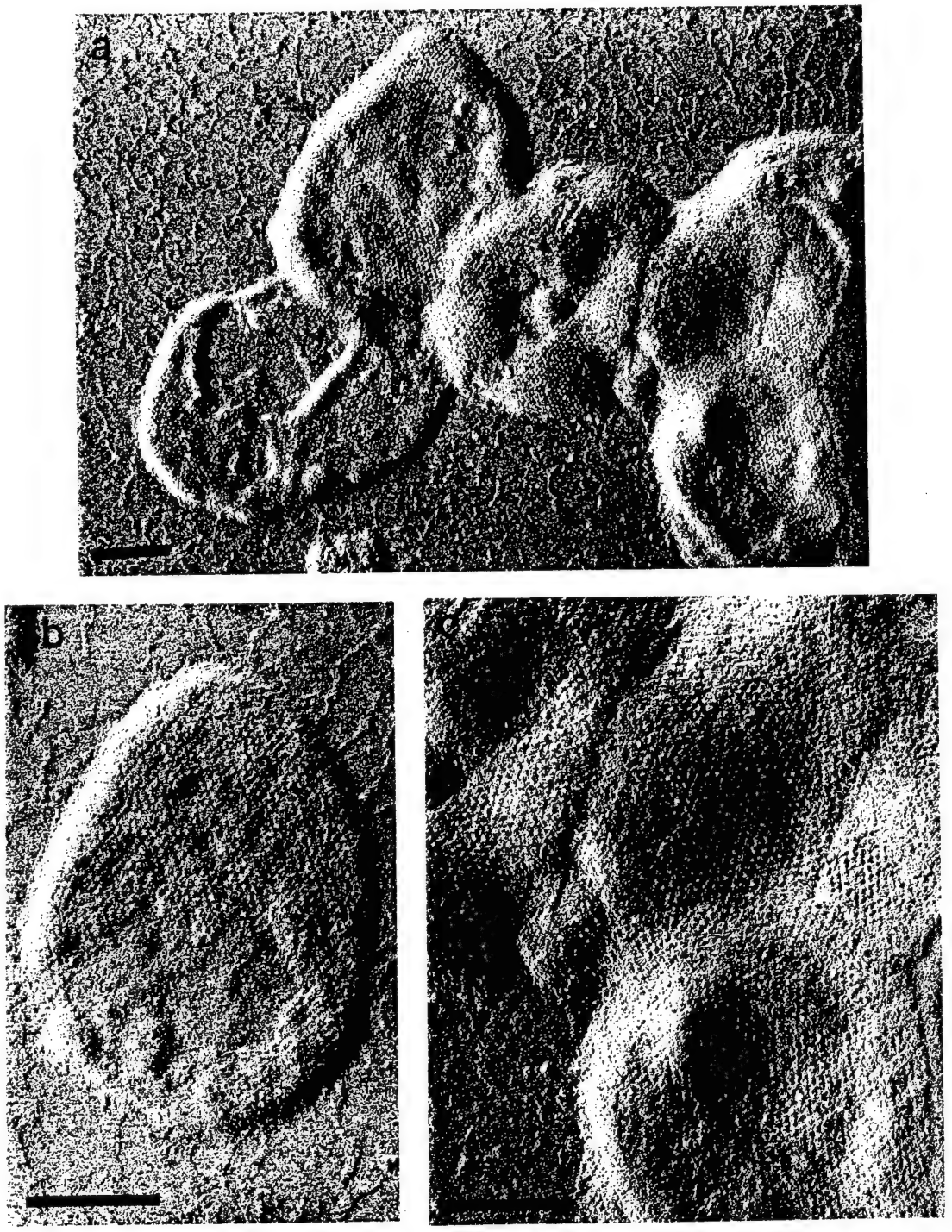
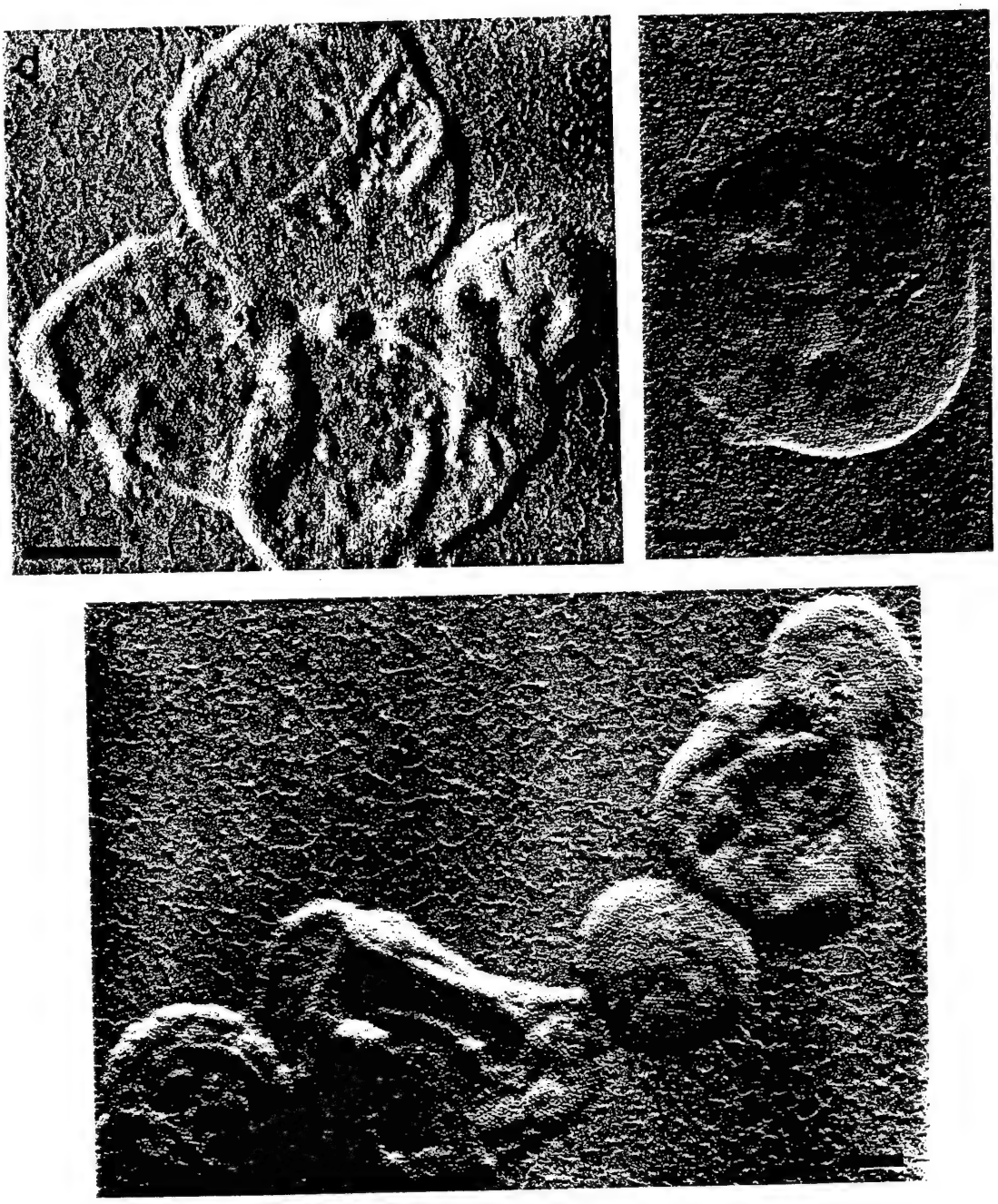


FIGURE 4.6 Platinum-replicas of *"D. ethenogenes"* strain 195 showing an hexagonal S-layer lattice. **(a)**, **(d)** and **(f)** show clusters of cells. **(b)** and **(e)** show single coccoid cells. **(c)** is a close up of the cells present to the right of the cluster depicted in **(a)**. All marker bars represent 0.3 μm.

FIGURE 4.6 (Continued)



observed in Fig. 3.11, the S-layer was located at the outermost part of the cell envelope.

4.5 DISCUSSION.

"D. ethenogenes" strain 195 was further characterized. The determination of an optimal dechlorinating temperature and pH is of environmental relevance. Most of the temperatures in which reductive dechlorination should occur in the environment are not above 15°C and pH values are often near neutrality in contaminated sites (5, 6). The optimal dechlorinating temperature for *"D. ethenogenes"* strain 195 ranges from 32 to 35°C, which falls in the interval of temperatures found for other PCE dechlorinating isolates, though this is a more restricted interval than, for example, the one for Strain TT4B (8) or *D. multivorans* (11), which can optimally dechlorinate PCE at lower temperatures. The fact that rates of dechlorination at 15°C doubled in a few weeks is indicative of the need for *"D. ethenogenes"* strain 195 to acclimate to temperatures different than 35°C, the one at which it has been growing for years. It is possible, then, that the range of optimal dechlorinating temperatures could widen to encompass a region closer to 25°C.

Even though there seems to be a tendency for the optimal pH intervals to be narrower than those for temperatures in the PCE dechlorinating isolates, this is not the case for *"D. ethenogenes"* strain 195. This organism has an optimal pH interval from 6.8 to 7.5, although rapid rates of dechlorination can still be obtained at pHs

from 6.3 to 7.9. The fact that the culture showed a short lag phase before starting to dechlorinate when growing at pH 7.9 may indicate again that *"D. ethenogenes"* strain 195 is an organism with ability to acclimate to new environmental conditions, given sufficient time.

When cultures of *"D. ethenogenes"* strain 195 were exposed to oxygen and then were re-reduced to the original optimal growth conditions, no PCE dechlorination occurred. The fact that no reductive dechlorination occurred in the presence of oxygen was expected because this type of reaction occurs only under anaerobic conditions (19). The lack of dechlorination after re-reduction indicates the requirement by *"D. ethenogenes"* strain 195 for anaerobic conditions. On the other hand, the enzymatic apparatus necessary to dechlorinate could have been irreversibly repressed or inactivated by oxygen. If this were true, no dechlorination could be possible even if *"D. ethenogenes"* strain 195 was not a strictly anaerobic organism.

The studies regarding the osmolality of the growth medium utilized by *"D. ethenogenes"* strain 195 are of importance for two main reasons: (a) so far, cells of *"D. ethenogenes"* strain 195 have been growing in a medium of low osmolality (from 80 to 110 mOsm) when compared to that of animal cells (350 mOsm) or bacterial cells lacking a peptidoglycan cell wall, like acholeplasmas and other mycoplasmas (from 290 to 350 mOsm) (12). (b) Also, when PCE and the other chloroethenes are reduced by hydrogen, one proton and one chloride ion are produced for each chloride substituted. The protons acidify the medium (this is why a buffer system is so important) and the chloride ions, together with the excess of sodium ions present,

augment the tonicity of the medium to high levels after many PCE doses ($HCl+NaHCO_3=CO_2+H_2O+NaCl$).

To find an optimal osmolality for the growth of *"D. ethenogenes"* strain 195 could mean better rates of PCE dechlorination and, in fact, this is what it is seen in the sequence of Figs. 4.4 and 4.5 where, after a short acclimation period, osmolalities closer to 300 mOsm allow faster PCE dechlorination than the usual around 100 mOsm. The fact that osmolalities of 430 mOsm allowed much poorer dechlorination rates than the rest means that lower osmolalities are supported much better by the culture than higher ones and, as with the temperature curve, there is a sharp decrease in activity once the optimal osmolality is exceeded.

Electron microscopy studies clearly showed the presence of a two-dimensional, crystalline bacterial S-layer present in the cell envelope of *"D. ethenogenes"* strain 195, which can be unequivocally identified only by electron microscopy (2). Not much is known about the significance of S-layers' function in cells (17). Some archaeobacteria possess S-layers as an exclusive wall component which determines cell shape (16, 17). This does not seem to be the case in *"D. ethenogenes"* strain 195. This organism lacks peptidoglycan but it does not have a definite, constant coccoid shape. The lack of rigidity of this organism can be seen in common folding of cell portions onto themselves when observed in microscopical preparations (see Chapters Three and Four, and Appendix A); and in flattening of cells that acquire a disc shape when laid onto an agar-coated slide, ncreasing their diameter by about 0.2 μm (Fig. 2.9).

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CHAPTER FIVE

SUBSTRATE UTILIZATION BY *"Dehalococcoides ethenogenes"* strain 195

"Just when you though it was safe to go back in the water"
Advertisement for the 1978 film Jaws 2.

5.1 ABSTRACT.

A study of the utilization of chlorinated ethenes and 1,2-dichloroethane (DCA) by "*Dehalococcoides ethenogenes*" strain 195 is presented. PCE grown "*D. ethenogenes*" strain 195 could be readily transferred into media amended with TCE, *cis*-DCE, 1,1-DCE or DCA as sole electron acceptor. The culture consumed these chlorinated ethenes and DCA faster with time, indicative of growth on these compounds. "*D. ethenogenes*" strain 195 dechlorinated 1,1-DCE faster than any other compound tested, followed by TCE, PCE, and DCA, and showed limitation of dechlorination when grown on *cis*-DCE. This organism could not be transferred on VC or *trans*-DCE when these substances were used as sole electron acceptors, but the culture could dechlorinate either if given several previous doses of PCE (most probably through a cometabolic process). Cultures grown on PCE, TCE, 1,1-DCE or DCA could immediately dechlorinate another of these chloroethenes, although some combinations proved to be dechlorinated faster than others.

5.2 INTRODUCTION.

The isolation and characterization of "*Dehalococcoides ethenogenes*" strain 195, a novel eubacterium capable of completely dechlorinating PCE to ETH, was described in Chapters Three and Four of this dissertation. This microorganism has a very limited range of utilizable substrates, is a small pleomorphic coccus with an optimal growth temperature of 35°C and optimal pH of 6.8 to 7.5 and lacks a peptidoglycan cell wall, having instead an S-layer type cell envelope (11 and Chapter Four of this dissertation). "*D. ethenogenes*" strain 195 could be transferred indefinitely into H_2/PCE medium supplemented with a mixture (called ABSS) of 2 mM acetate, 0.05 mg/l vitamin B_{12}, and 25% v/v anaerobic digestor sludge supernatant and 5% v/v 50x concentrated cell extract from a butyrate/PCE culture (11, 12).

Up to this point, all studies performed on "*D. ethenogenes*" strain 195, both in the mixed H_2/PCE culture (12) and in the pure culture (11, Chapter Four of this dissertation), have been accomplished by amending the medium with only one chloroethene, PCE, as the primary cell electron (e^-) acceptor for growth. Countless transfers of "*D. ethenogenes*" strain 195 on PCE and past studies (11, 12) have already ascertained the importance and the patterns of utilization of this molecule by this microorganism. Nevertheless, it is of environmental significance to compare the utilization of PCE relative to the utilization of the other chloroethenes by the same axenic culture. Similar studies were performed on the mixed methanol/PCE enrichment culture by Tandoi *et al.* (18). In their

studies, PCE, TCE, *cis*-DCE and 1,1-DCE were metabolized to VC with zero-order kinetics in the high concentration ranges measured, while *trans*-DCE and VC were dechlorinated with first-order kinetics. In addition, it was reported that the culture was also capable of rapidly dechlorinating 1,2-dichloroethane (DCA) and 1,2-dibromoethane (DBA).

As described in Chapter One of this dissertation, several other cultures that reductively dechlorinate PCE have been isolated very recently, but all these organisms dechlorinate PCE and TCE mainly to *cis*-DCE (6, 7, 9, 13, 16, 17). Therefore, there is an inherent lack of information about how the other DCE isomers and VC are utilized by pure cultures. In addition, very little is known about the versatility of these isolates in dechlorinating other chloroaliphatics and to switch from one chloroethene to another.

In this chapter, a study of the utilization of various chlorinated ethenes as substrates for dechlorination to ETH by *"D. ethenogenes"* strain 195 is presented. Studies on the reductive dechlorination of DCA by this culture and its relation to chloroethene degradation are also presented. Dechlorination of *cis*-DCE and VC will be dealt with separately in the next two chapters.

5.3 MATERIALS AND METHODS.

5.3.a Chemicals and analyses of chloroethenes.

PCE and other chlorinated ethenes, ETH, H_2, and other chemicals were purchased and utilized as described in Section 2.3.a of

this dissertation. DCA was purchased from Aldrich Chemical Co. (Milwaukee, WI). For quantitative analysis of chloroethenes, DCA and ETH, gas samples were analyzed using a Perkin Elmer Gas Chromatograph 8500, as described in Section 3.3.b of this dissertation.Headspace samples were 100-µl in all cases.

5.3.b Growth medium and culture conditions.

The basal salts medium utilized for *"D. ethenogenes"* strain 195 contained the same ingredients and was produced as described in Chapter Two of this dissertation. This medium received the amendments described in Chapter Three of this dissertation, with the addition of 5% v/v extract from the butyrate/PCE culture (prepared as described in Chapter Three). The antibiotic ampicillin, added at 0.3 µg/ml to the cultures, was prepared as described in Chapter Three.

Unless otherwise stated, inoculum sizes were 2% v/v, all incubations were done in duplicate, and each experiment presented was performed at least twice with similar results. Duplicate tubes performed very similarly in most of the experiments presented (less than 5% difference in results for tubes under same conditions), so the results for some of the experiments are presented for individual tubes. Cultures were incubated as described in Chapter Two.

5.4 RESULTS.

5.4.a Use of PCE, TCE, DCE isomers, VC and DCA as primary electron acceptors.

Fig. 5.1 shows the results of an experiment in which a culture of *"D. ethenogenes"* strain 195 grown on PCE was transferred into sets of tubes containing, separately, each of the chlorinated ethenes and DCA as sole e^- acceptor sources. 1,1-DCE was dechlorinated faster than the other substrates (1.3±0.12 mmol VC+ETH produced l^{-1} day^{-1}; days 5-6.5). TCE and PCE were dechlorinated at similar rates (0.93±0.08 mmol VC+ETH l^{-1} day^{-1} for TCE; days 6-7.5; and 0.89±0.055 mmol VC+ETH l^{-1} day^{-1} for PCE; days 6.5-8), which were a little higher than the one for DCA dechlorination (0.64±0.05 mmol ETH l^{-1} day^{-1}; days 5-6.5), a process which produced very little VC (about 0.019 mmol/l in 6.5 days; data not shown). *cis*-DCE was dechlorinated more slowly than the above mentioned substrates (0.056±0.03 mmol VC l^{-1} day^{-1}; days 6.5-8). As seen also in Fig. 5.2, VC and *trans*-DCE were dechlorinated to a very small extent with no rate increase. *trans*-DCE produced 0.019 mmol/l of VC in 14 days from an initial dose of 0.32 mmol/l of *trans*-DCE. No ETH was produced from *trans*-DCE. VC produced 0.0085 mmol/l of ETH in 14 days from an initial dose of 0.35 mmol/l of VC.

In Fig. 5.1, cultures grown with PCE, TCE, and 1,1-DCE were not fed for two days after their second chloroethene dose had been consumed, to observe ETH production (data not shown), hence the lack of net VC production during that time. Cultures that didn't have

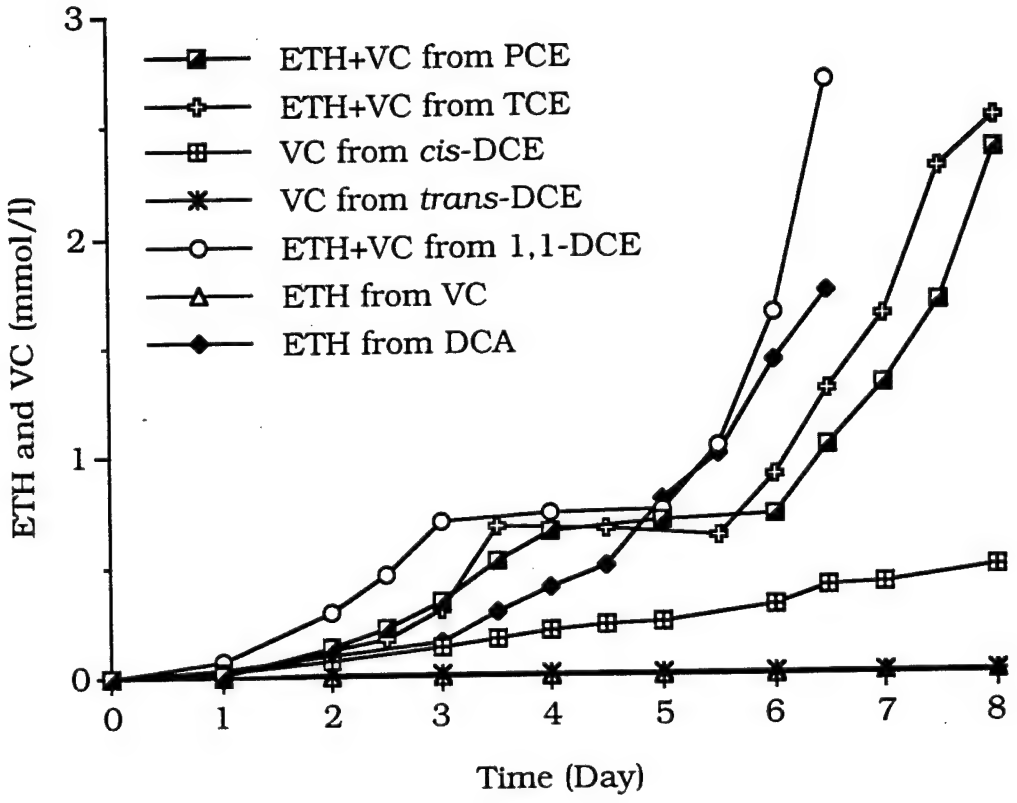

FIGURE 5.1 Product (ETH and/or VC) formation by *"D. ethenogenes"* strain 195 grown with PCE and inoculated into medium containing the next substrates, tested separately (in parenthesis are shown the total amounts of substrate consumed by the cultures, in mmol/l): PCE (2.5); TCE (2.5); *cis*-DCE (0.67); *trans*-DCE (0.07); 1,1-DCE (2.85); VC (0.17); and DCA (1.8).

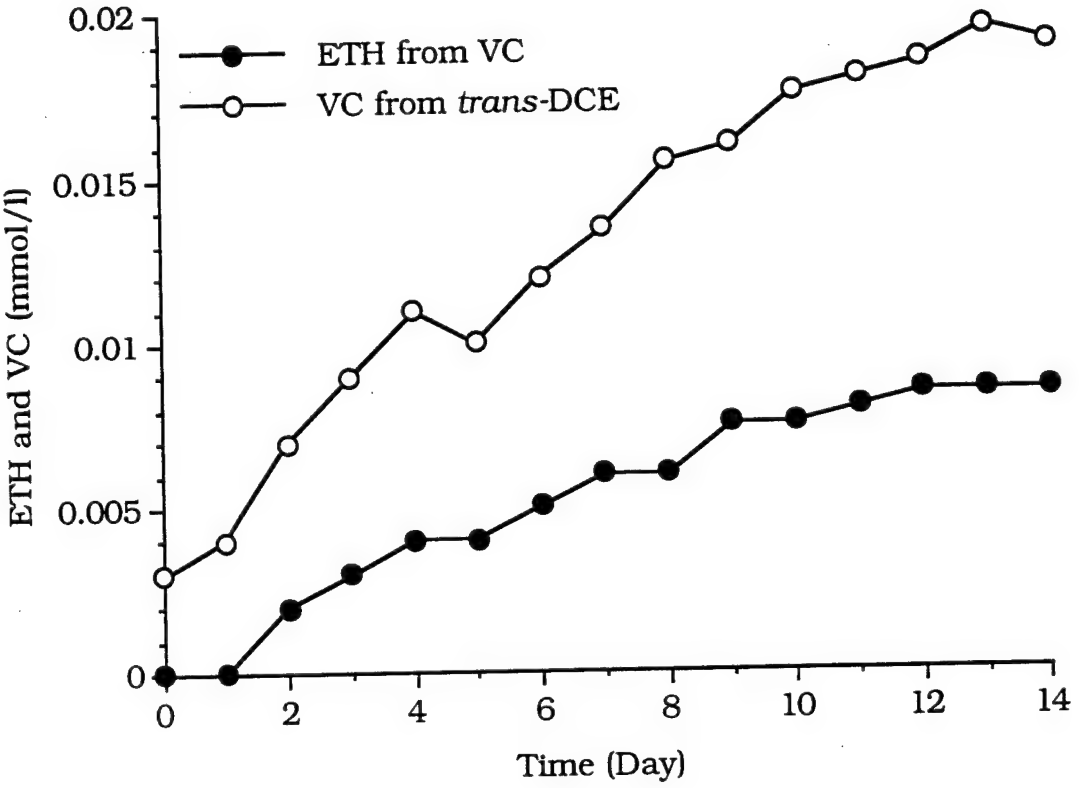

FIGURE 5.2. Amounts ETH and VC produced from VC and *trans*-DCE (respectively) in the experiment depicted in Fig. 5.1.

substrate withheld for two days were the ones amended with: DCA, which produced almost exclusively ETH; *cis*-DCE, which did not finish their second chloroethene dose; and *trans*-DCE and VC, which did not finish their first chloroethene dose.

5.4.b PCE metabolism to ETH. Intermediate formation.

Fig. 5.3 (a) shows the results of an experiment in which a high initial dose of PCE (0.7 mmol/l) was administered to tubes that had been inoculated with a culture that was very actively dechlorinating PCE to VC and ETH. As it has been shown previously, PCE was converted mainly to VC, and VC conversion to ETH occurred after PCE depletion. Fig. 5.3 (b) shows, with greater sensitivity, the sequence and magnitude in which these intermediates were accumulated. TCE was present within 2 hours of inoculation and was produced before any DCE isomer. Then, *cis*-DCE, 1,1-DCE and *trans*-DCE appeared in that order before 15 hours had elapsed. Once PCE had been consumed at about 72 hours, the intermediates rapidly disappeared, except *trans*-DCE, which persisted for the duration of the experiment.

5.4.c TCE metabolism to ETH-intermediate formation.

"D. ethenogenes" strain 195 was able to grow with TCE as sole e^- acceptor (Fig. 5.1) and could be transferred on the same substrate without showing any acclimation phase, as it can be seen in Fig. 5.4. The sequence and magnitude of the intermediates formed from an initial dose of TCE (0.35 mmol/l) added to a culture of *"D.*

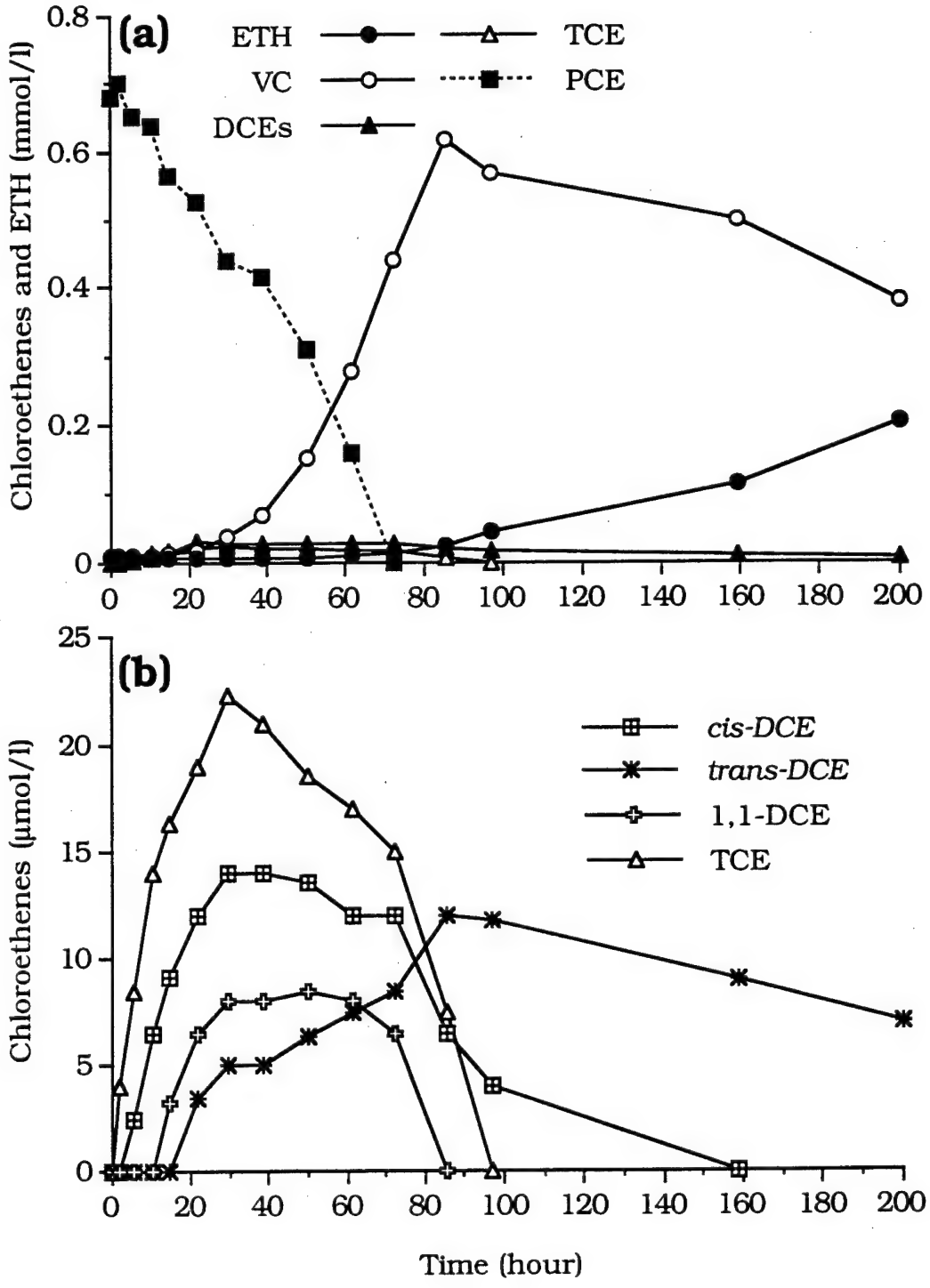

FIGURE 5.3 (a) Product formation by *"D. ethenogenes"* strain 195 in medium to which one high dose of PCE (0.7 mmol/l) was added. **(b)** Sequence and magnitude in which the intermediate products of PCE metabolism were produced and depleted by the same culture.

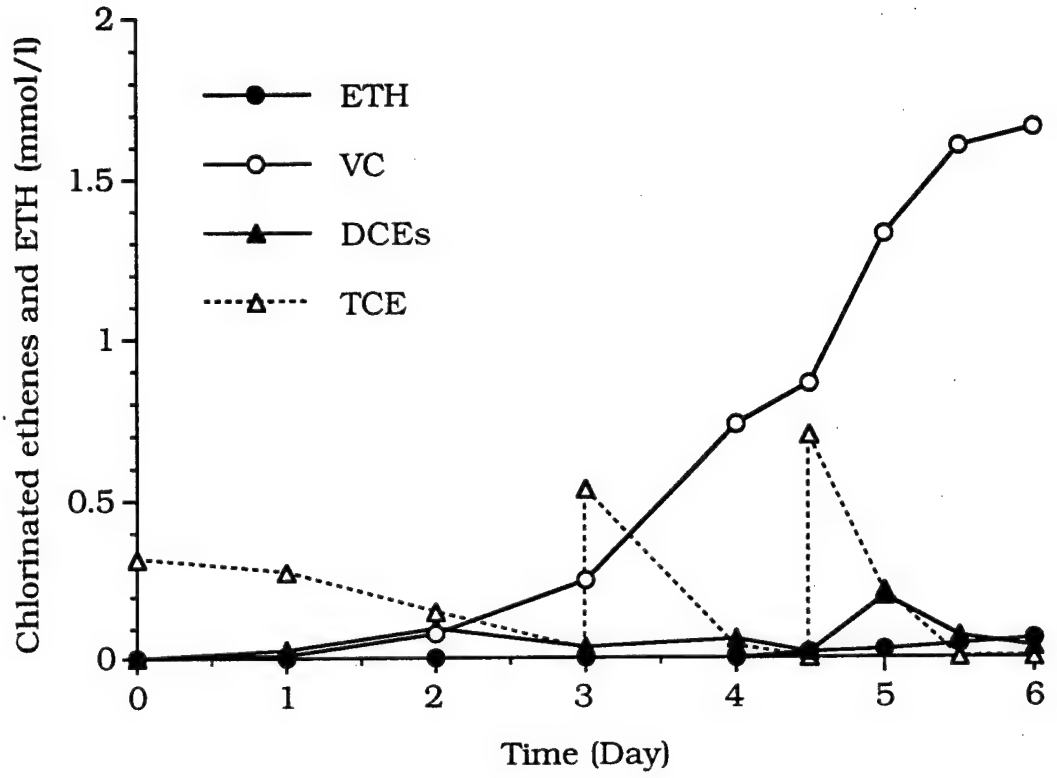

FIGURE 5.4 Product formation by *"D. ethenogenes"* strain 195 after being transferred on TCE as sole electron acceptor for a second time. The graph shows the first three TCE feedings to the culture.

ethenogenes" strain 195 is shown in Fig. 5.5. *cis*-DCE was also produced before the other two DCE isomers. While *trans*-DCE and 1,1-DCE accumulated to similar levels than in Fig. 5.3, *cis*-DCE accumulated to much higher levels (about 7 times higher than that produced in Fig. 5.3). *trans*-DCE, once produced, persisted again for the duration of the experiment. As in Fig. 5.3, the decrease in the amount of *trans*-DCE over time was consistent with the amount of any DCE isomer that was normally lost by absorption/diffusion in/through the stopper (data not shown).

An experiment was performed to test the ability of "*D. ethenogenes*" strain 195 to dechlorinate PCE after having consumed several doses of TCE as sole e^- acceptor. TCE is an intermediate in the PCE dechlorination pathway to ETH, but PCE is not an intermediate in TCE dechlorination, and it was possible that TCE-grown cells could not have the ability to use PCE. At day 8, the culture grown on TCE from Fig. 5.1 was transferred and fed again with TCE, three consecutive doses (0.3, 0.5 and 0.7 mmol/l TCE). Fig. 5.6 shows the results of the fourth dose, which was of TCE in (a) and PCE in (b). After a total of 14 days and seven TCE doses administered, the culture was able to use PCE without any acclimation phase. During the fourth dose, the rates of product formation were 1.08±0.10 mmol l^{-1} day^{-1} for PCE and 0.81±0.06 mmol l^{-1} day^{-1} for TCE.

5.4.d 1,1-DCE utilization.

"*D. ethenogenes*" strain 195 was able to dechlorinate 1,1-DCE as sole e^- acceptor at a high rate (Fig. 5.1) and could be transferred on

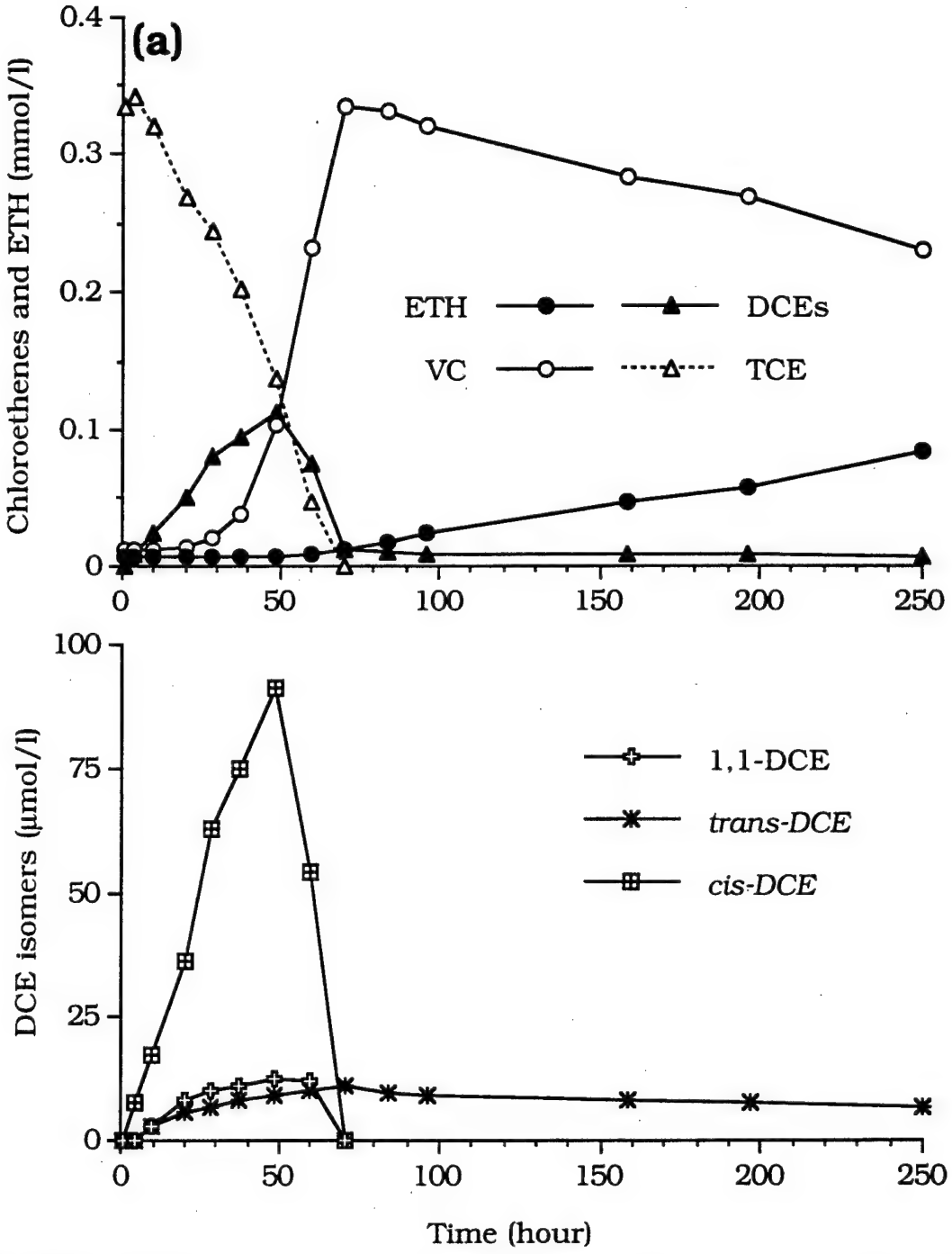

FIGURE 5.5 (a) Product formation by *"D. ethenogenes"* strain 195 inoculated into medium containing all requirements for growth, in which a single dose of 0.35 mmol/l TCE was added instead of PCE. **(b)** Sequence and magnitude in which the different intermediate products of TCE metabolism were produced and depleted by the same culture.

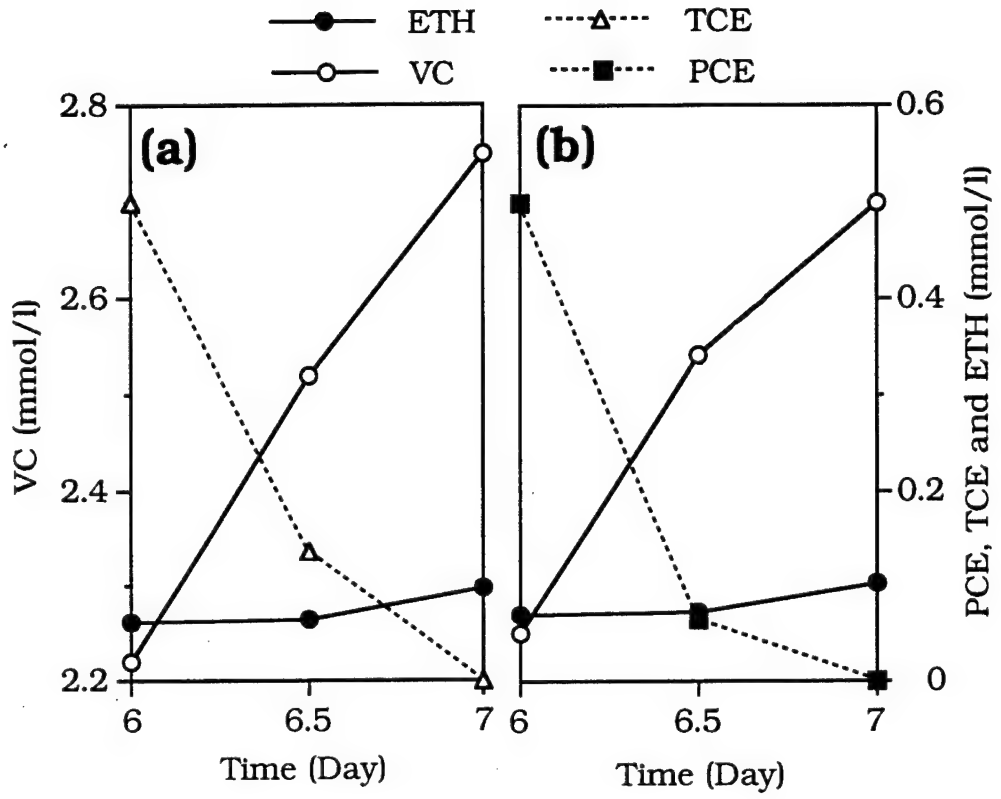

FIGURE 5.6 TCE **(a)** and PCE **(b)** disappearance and product formation by *"D. ethenogenes"* strain 195. Cultures were grown and transferred with TCE as sole electron donor. After being transferred, in both experiments {**(a)** and **(b)**} TCE was fed three consecutive times (0.3, 0.5 and 0.7 mmol/l). The fourth feedings {TCE in **(a)** and PCE in **(b)**} are the ones shown in this figure.

the same substrate without showing any acclimation phase (Fig. 5.7).

An experiment was performed to test the ability of 1,1-DCE-grown *"D. ethenogenes"* strain 195 to dechlorinate chlorinated ethenes with the same or higher number of chlorines (except *trans*-DCE). At day 6, the culture grown on 1,1-DCE from Fig. 5.1 was transferred and fed again with 1,1-DCE, three consecutive doses (0.3, 0.5 and 0.7 mmol/l). Fig. 5.8 shows the results of the fourth dose, which was of 1,1-DCE in (a), PCE in (b), TCE in (c) and *cis*-DCE in (d). After 11 days and seven 1,1-DCE doses administered, the culture was able to switch to the other chlorinated ethenes tested without any acclimation phase. During days 5 to 5.5, PCE was dechlorinated at 0.63±0.07 mmol l^{-1} day^{-1} and TCE at 0.71±0.06 mmol l^{-1} day^{-1}. The rates for 1,1-DCE (0.80±0.085 mmol l^{-1} day^{-1}) and *cis*-DCE (0.84±0.04 mmol l^{-1} day^{-1}) were very similar.

5.4.e *trans*-DCE utilization.

The results shown in Fig. 5.9 corroborate the findings of Figs. 5.3 and 5.4, in which *trans*-DCE, once produced by the culture, was not dechlorinated to any significant extent. The culture utilized for this experiment was a unusually slow growing culture, a factor that helped to see more clearly this effect (the reason for the slow down in the activity of this culture will be hypothesized in Appendix A of this dissertation). During the time that PCE was absent from the medium (days 12 to 13 and from day 15 onwards), the culture was able to dechlorinate all the accumulated intermediates, except *trans*-DCE, to VC and ETH (data not shown for VC and ETH), reaffirming the

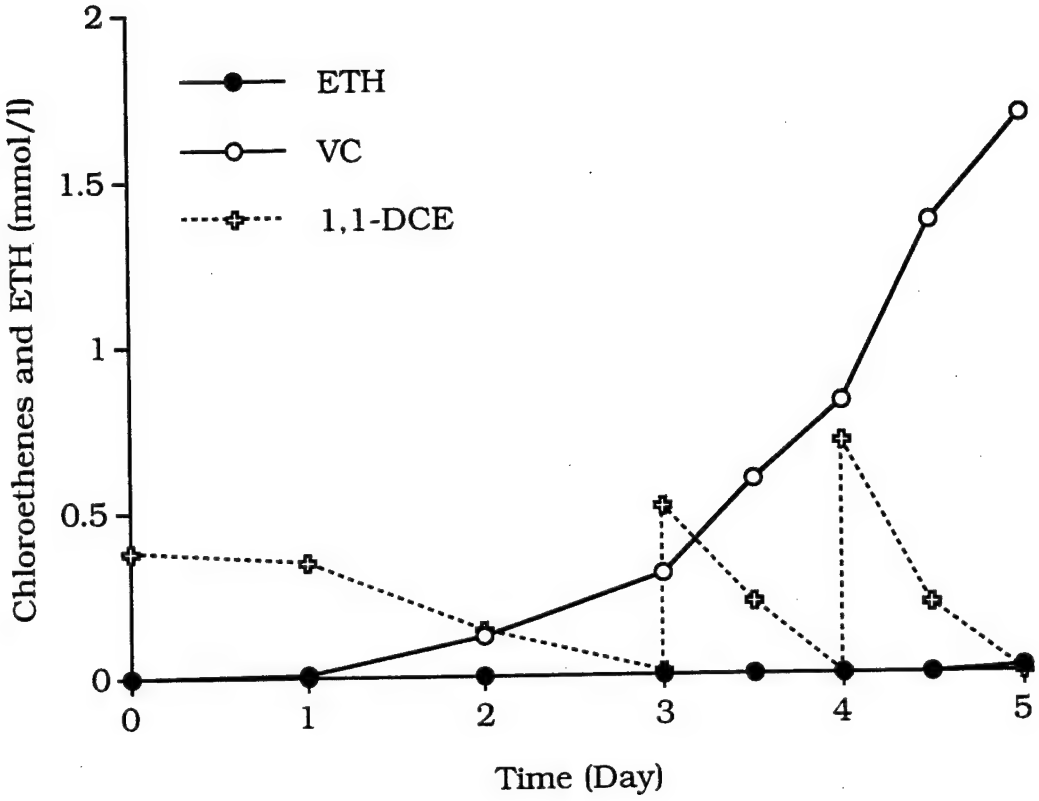

FIGURE 5.7 Product formation by *"D. ethenogenes"* strain 195 after being transferred on 1,1-DCE a second time as sole e^- acceptor. The graph shows the first three 1,1-DCE feedings to the culture.

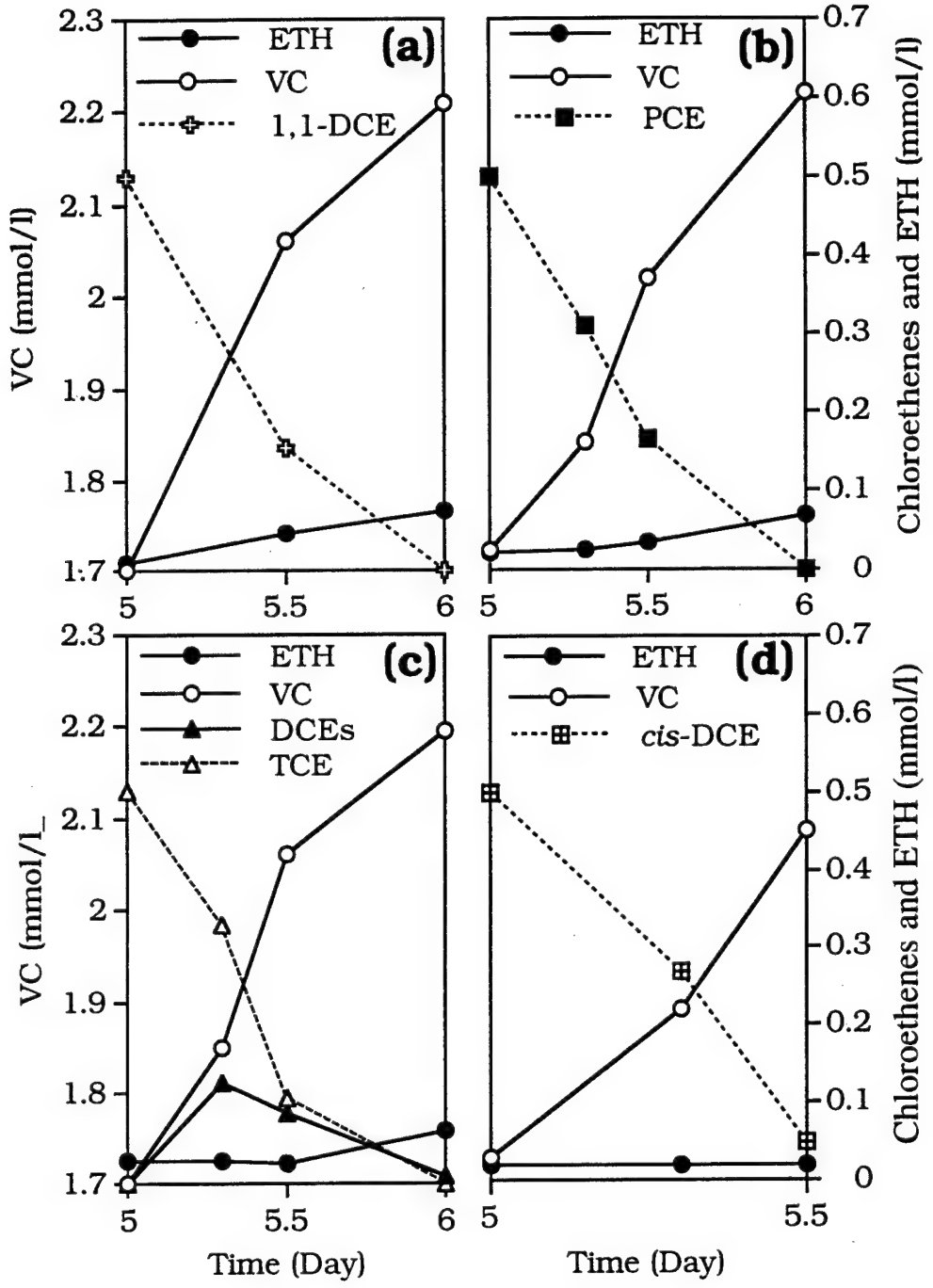

FIGURE 5.8 1,1-DCE **(a)**, PCE **(b)**, TCE **(c)** and *cis*-DCE **(d)** disappearance and product formation in cultures of *"D. ethenogenes"* strain 195 transferred with 1,1-DCE as sole electron acceptor. In all experiments, 1,1-DCE was fed in three consecutive doses (0.3, 0.5 and 0.7 mmol/l). The fourth doses {0.5 mmol/l of 1,1-DCE in **(a)**, PCE in **(b)**, TCE in **(c)** and *cis*-DCE in **(d)**} are the ones shown.

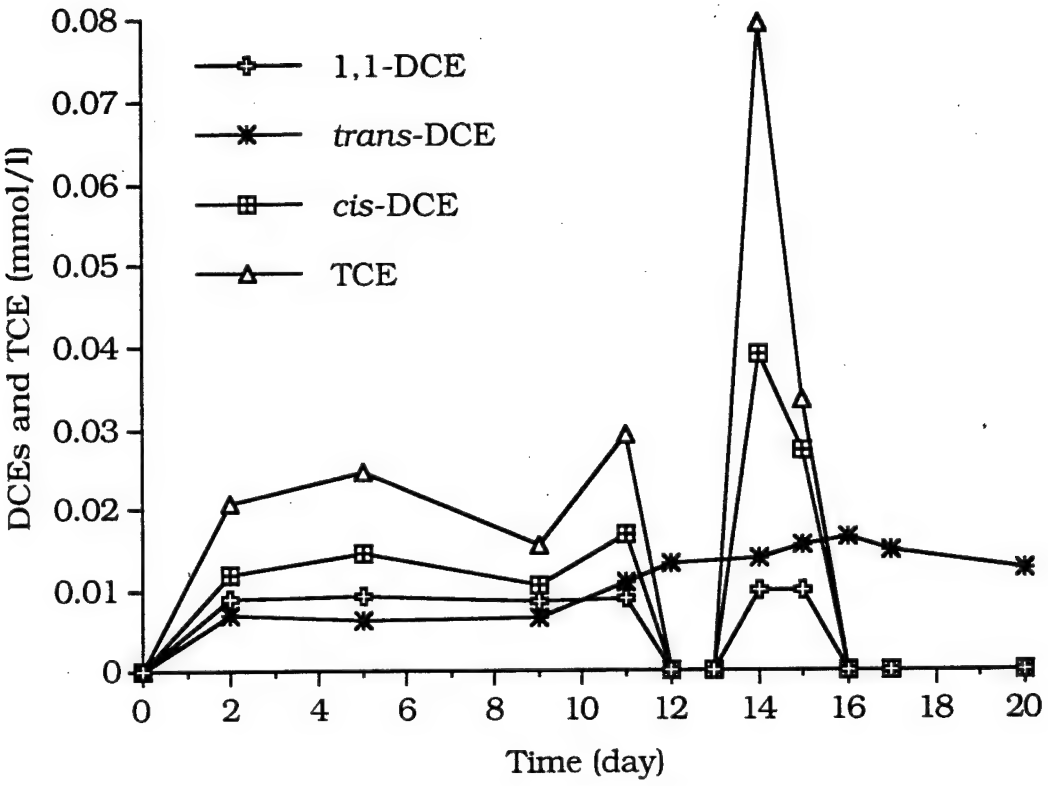

FIGURE 5.9 DCE isomers and TCE produced and consumed by a slow growing culture of *"D. ethenogenes"* strain 195. PCE was absent at days 12 to 13 and from day 15 onwards. PCE, VC and ETH have not been plotted for clarity.

inability of *"D. ethenogenes"* strain 195 to effectively dechlorinate *trans*-DCE, even though this compound was produced by the culture.

The recalcitrance of *trans*-DCE could be due to the inability of the culture to reductively dechlorinate this molecule or to the impossibility of *"D. ethenogenes"* strain 195 to obtain energy for growth from *trans*-DCE. The results of Fig. 5.10 (a) show that, when a culture was fed three consecutive PCE doses, a fourth high dose of *trans*-DCE (0.35 mmol/l) was dechlorinated to 0.015 mmol/l in 5.5 days. The dechlorination rate (measured as ETH production) for the first 1.5 days was of 0.092 mmol l^{-1} day^{-1}, which decreased to 0.027 mmol l^{-1} day^{-1} in the next 2.5 days, and to 0.010 mmol l^{-1} day^{-1} in the final 1.5 days. Fig. 5.11 depicts a semilogarithmic plot of *trans*-DCE disappearance produced from the values obtained in Fig. 5.10 (a). The plot yielded a straight line (r^2 = 0.996), indicating first-order kinetics and an estimated half-life of 28.8 hours. When the culture was autoclaved at day 5, no *trans*-DCE dechlorination was effected {Fig. 5.10 (b)}.

An interesting aspect derived from the results shown in Fig. 5.10 (a) is that *trans*-DCE did not inhibit ETH production from VC, as the other chlorinated ethenes did (data shown in Chapter Seven). Also, the rates of ETH formation from VC seemed to be similar to the rates of VC formation from *trans*-DCE. This produced the effect that *trans*-DCE seemed to be transformed directly to ETH when, most probably, *trans*-DCE was dechlorinated to VC, and then to ETH.

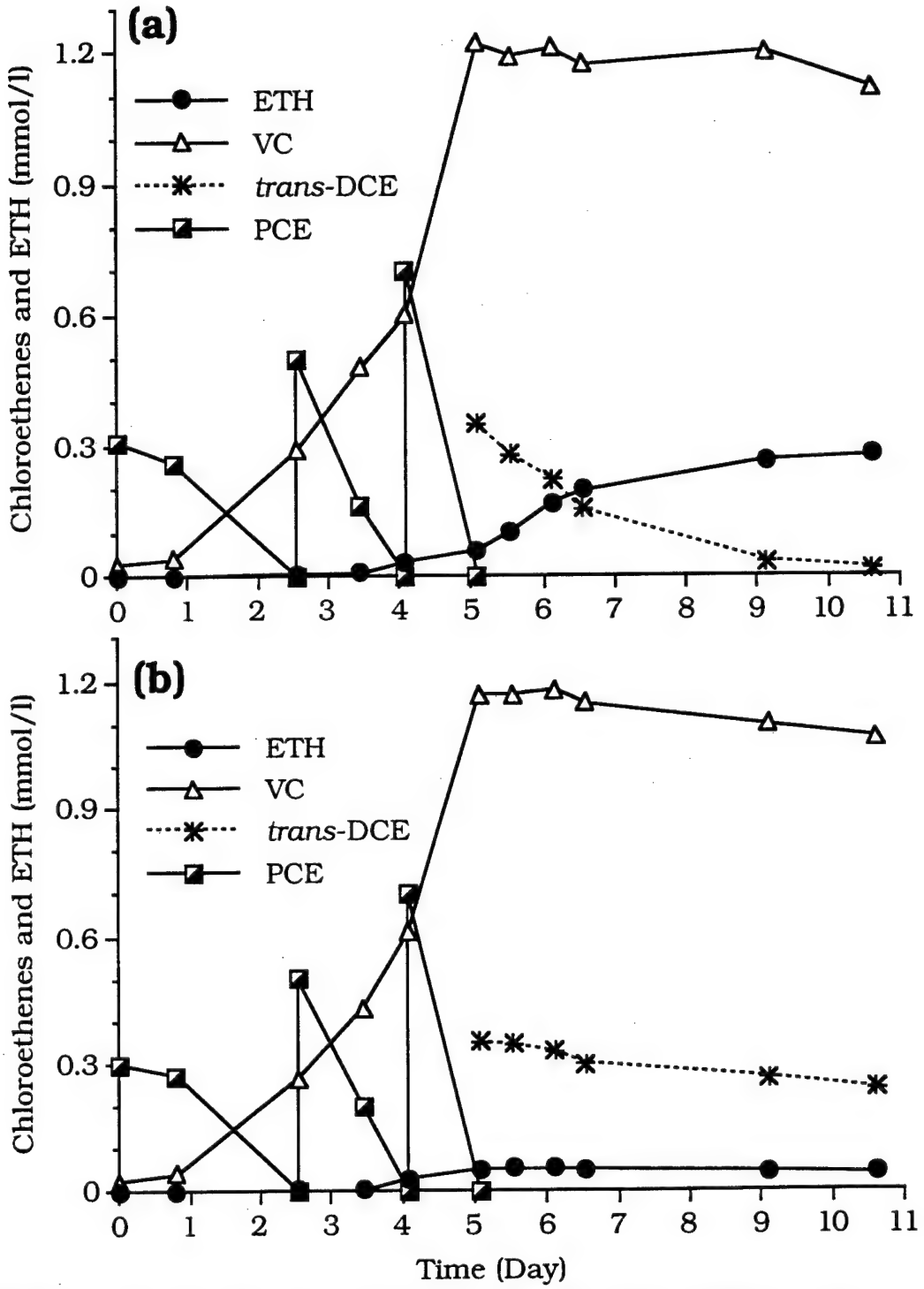

Figure 5.10 (a) Effect on product formation after adding a dose (0.35 mmol/l) of *trans*-DCE after feeding the culture with three consecutive doses of PCE (0.3, 0.5 and 0.7 mmol/l). **(b)** Same conditions as in **(a)**, but here the cultures were autoclaved at day 5.

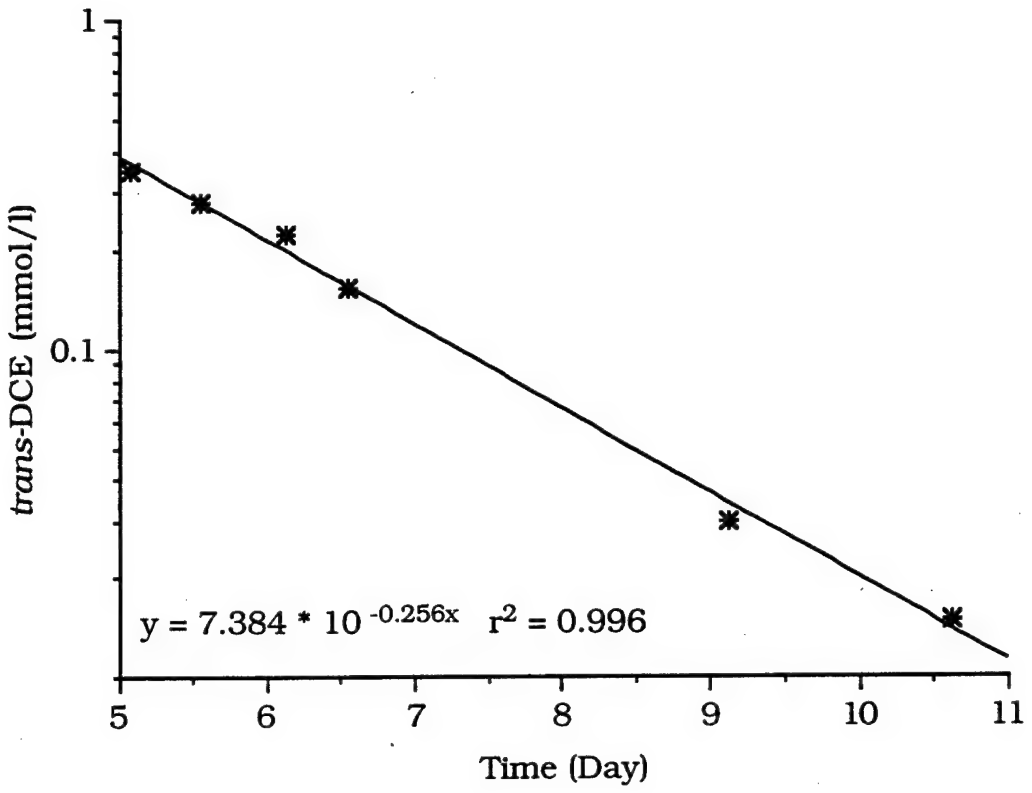

FIGURE 5.11 Semilogarithmic plot of *trans*-DCE disappearance. Data obtained from the experiment shown in Fig. 5.10 (a).

5.4.f DCA utilization.

"D. ethenogenes" strain 195 was able to dechlorinate DCA as sole e^- acceptor (Fig. 5.1) and could be transferred on the same substrate without showing any acclimation phase. Fig. 5.12 shows the almost complete dechlorination of DCA to ETH by a culture of *"D. ethenogenes"* strain 195 that had been growing on DCA as the sole e^- acceptor for 16 days. This culture had consumed eight DCA doses and it had been successfully transferred twice before this experiment was initiated. DCA was used at an increasing rate. VC production from DCA was about 1.3% of the ETH production, with the relative amounts remaining constant throughout the incubation.

An experiment was performed to test the ability of this organism to dechlorinate the chloroethenes (except VC and *trans*-DCE) after having consumed several doses of DCA as sole e^- acceptor. At day 7, the culture grown on DCA from Fig. 5.1 was transferred and fed with three consecutive doses (0.3, 0.5 and 0.7 mmol/l) of DCA. Fig. 5.13 shows the results of the fourth doses, which were of DCA in (a), PCE in (b), TCE in (c), *cis*-DCE in (d) and 1,1-DCE in (e). After a total of 14 days and seven DCA doses administered, the culture was able to switch to the other chlorinated ethenes tested without any acclimation phase. During the first 12 hours of the fourth doses, the rate of product formation (in mmol l^{-1} day^{-1}) were: from DCA 0.78±0.06; from PCE 0.61±0.08; from TCE 0.84±0.05; from *cis*-DCE 0.48±0.03; and from 1,1-DCE 0.48±0.05.

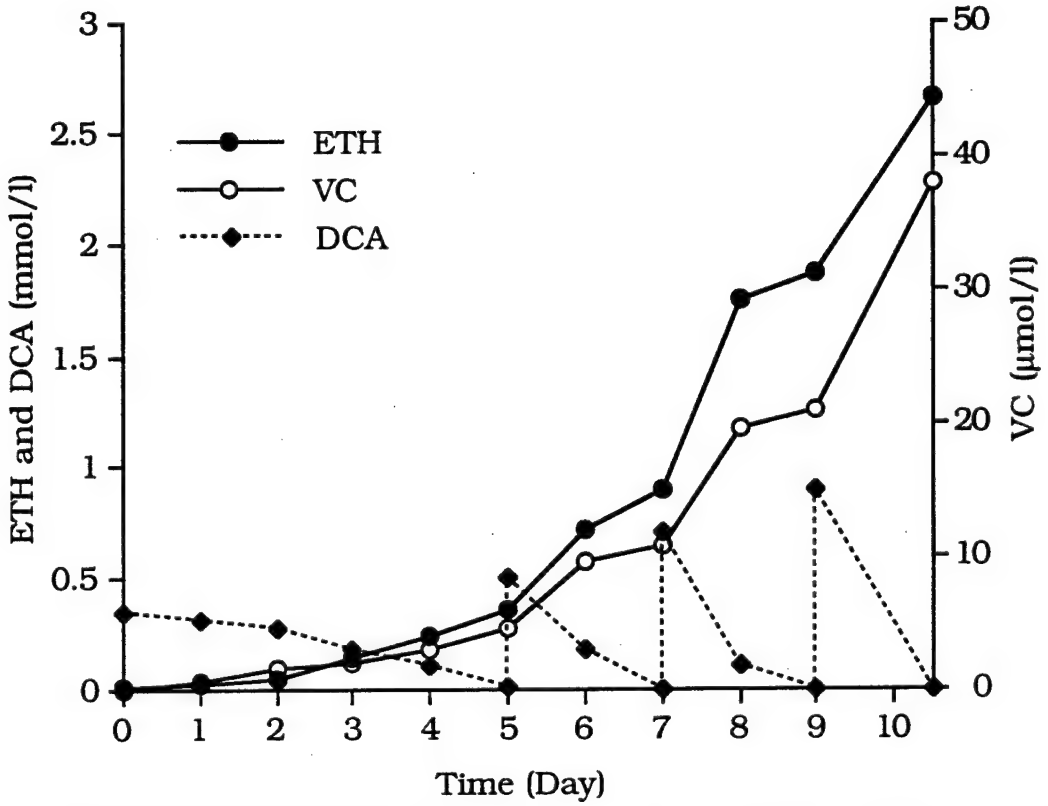

Figure 5.12 Product formation by *"D. ethenogenes"* strain 195 growing on DCA instead of PCE. The graph depicts a culture that had been transferred three times on DCA only. The inoculum utilized for this transfer had been at 4°C for two weeks, hence the relatively slow start of dechlorination. The amounts of VC produced are shown on the right Y axis (note the scale on this axis is in µmol/l).

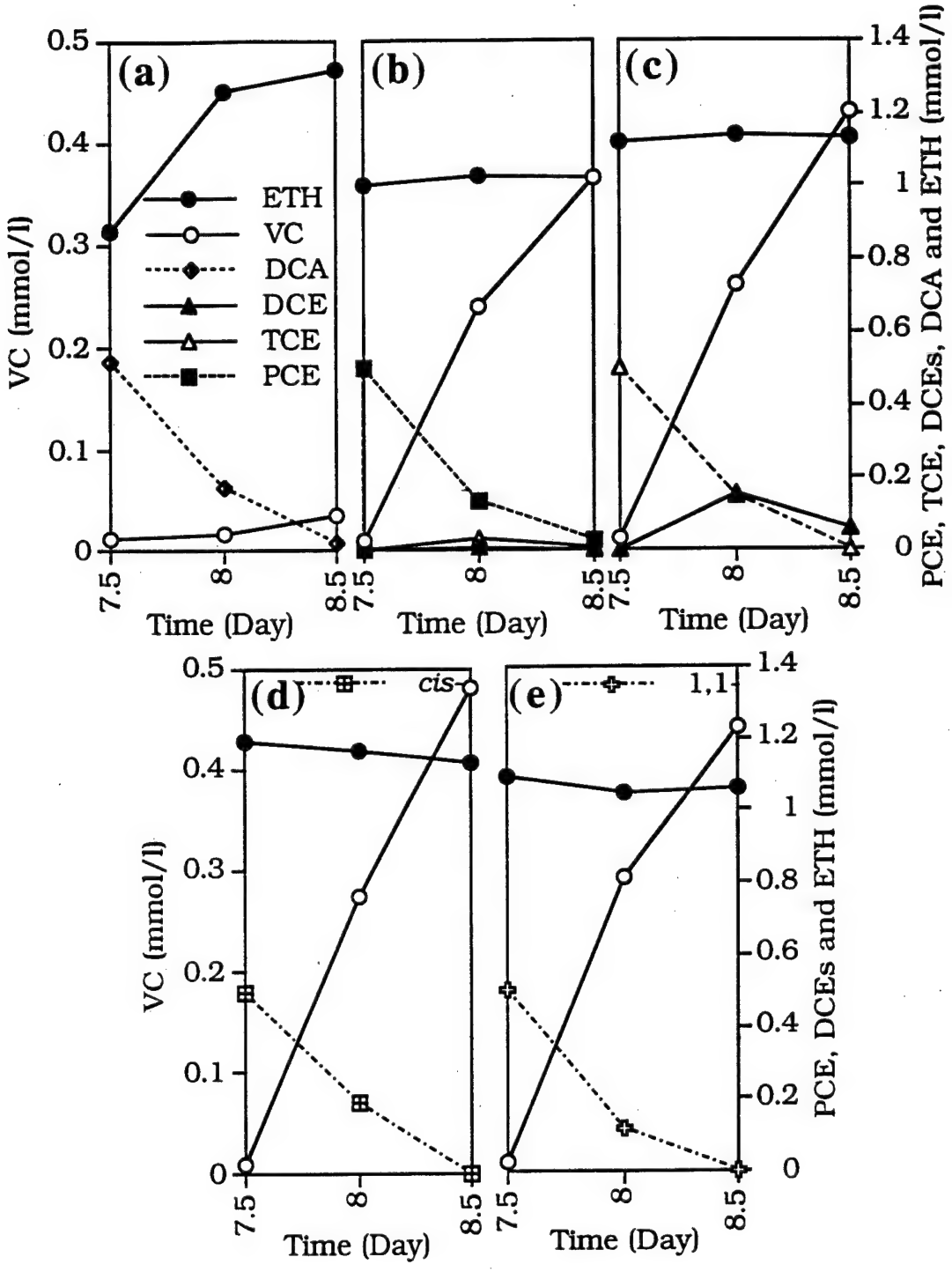

FIGURE 5.13 DCA **(a)**, PCE **(b)**, TCE **(c)**, *cis*-DCE **(d)**, and 1,1-DCE **(e)** disappearance and product formation in cultures transferred with DCA as sole e^- acceptor. In all experiments DCA was fed 3 times (0.3, 0.5 and 0.7 mmol/l). The fourth feedings {0.5 mmol/l DCA in **(a)**; PCE in **(b)**; TCE in **(c)**; *cis*-DCE in **(d)**; 1,1-DCE in **(e)**} are the ones shown.

5.5 DISCUSSION.

The comparison between the utilization patterns and rates of degradation of chloroethenes by *"D. ethenogenes"* strain 195 and the ability of this organism to switch from dechlorinating one chloroethene to another is of environmental relevance. This is so, both because of the inherent importance of an organism that is capable of complete PCE reduction, but also because more knowledge has to be accrued regarding how an ongoing biodegradation process will behave in answer to unpredicted changes in chloroethene type predominance and/or accumulation.

The PCE dechlorination pattern of Fig. 5.3 is congruent with PCE dechlorination to TCE with conversion of TCE primarily to *cis*-DCE, which was the first product to appear after TCE appearance. However, 1,1-DCE appeared soon afterwards and accumulated to amounts one-half to two-thirds of the amount of *cis*-DCE. Thus, we cannot rule out 1,1-DCE being a significant intermediate in TCE conversion to VC. *trans*-DCE accumulated slowly and was reduced even more slowly, if at all, suggesting that it represents only a small portion of substrate flow.

In earlier studies (18), it was shown that the mixed culture containing *"D. ethenogenes"* strain 195 could use all of the chlorinated ethenes as well as dichloroethane. However, that experiment simply asked whether pre-grown cultures were able to metabolize a dose of these compounds. It was of interest to examine whether *"D. ethenogenes"* strain 195 could grow on these compounds, and, if so, if the culture was still able to metabolize PCE, since it is possible that

cultures grown on less chlorinated ethenes, such as DCEs, might not be able to use a more chlorinated one.

"D. ethenogenes" strain 195 was capable of readily utilizing PCE, TCE, 1,1-DCE and DCA as sole e^- acceptors for growth, dechlorinating them to VC and ETH. *cis*-DCE could also serve as growth substrate, although it was utilized at a much lower rate than the other chlorinated ethenes and DCA. According to Fig. 5.1, the sequence in which the different chlorinated molecules were degraded as e^- acceptors, from faster to slower, was: 1,1-DCE>TCE>PCE>DCA>>*cis*-DCE>>*trans*-DCE=VC≈0. However, if these same dechlorination processes are calculated per e^- basis (number of e^- pairs reduced in each molecule), the order in which these substrates were dechlorinated, starting with the best, is changed: PCE>TCE>1,1-DCE>DCA>>*cis*-DCE>>>*trans*-DCE=VC≈0. DCE dechlorination to VC only involves a single e^- pair while PCE and TCE conversion to VC requires three and two e^- pairs respectively (and, theoretically, provides three and two times as much energy per VC produced). The fact that the rates of dechlorination of *cis*-DCE to VC and ETH drastically increased when a culture utilized this substrate as sole e^- acceptor after growing on 1,1-DCE or TCE implied a need for more studies on the distinctive behavior of *cis*-DCE. The particulars of *cis*-DCE dechlorination are described in Chapter Six of this dissertation.

"D. ethenogenes" strain 195 could not utilize VC or *trans*-DCE as growth substrates. Nevertheless, very slow, first-order *trans*-DCE dechlorination to VC and VC dechlorination to ETH occurred. In

other similar experiments the persistence of *trans*-DCE was repeatedly detected in the culture, which may explain the early result of Freedman and Gossett (5) on the methanol/PCE mixed culture in which *trans*-DCE was detected. The results in Fig. 5.10 indicated the ability of *"D. ethenogenes"* strain 195 to dechlorinate high concentrations of *trans*-DCE if a suitable substrate like PCE was previously administered as an e^- acceptor to built up biomass (which was also observed for VC; see Fig. 3.9 and Chapter Seven). This pattern of utilization of *trans*-DCE by *"D. ethenogenes"* strain 195 seems to be typical of a cometabolic process, in which no energy is obtained from the compound, but the molecule in question is dechlorinated when a suitable growth substrate is utilized. The facts that the rates of *trans*-DCE disappearance diminished with time (first-order kinetics) when PCE was not present (Fig. 5.10) and that there was no increase in the rates of product formation from *trans*-DCE (Fig. 5.1) reinforce the view that *trans*-DCE dechlorination is, most probably, cometabolic.

An interesting observation from the experiment in Fig. 5.10 (a) is that, contrary to the effect of PCE (11, 12), *trans*-DCE dechlorination did not inhibit ETH formation from VC, which is in accordance with the results found for the mixed enrichment culture by Tandoi *et al.* (18).

The reason why VC and *trans*-DCE are not utilized as energy sources is not known. A characteristic shared by both molecules is that they lack adjacent chlorines. This factor could be of importance

in regard to the active sites of dehalogenating enzymes, but very little is presently known about these dehalogenases (4, 8, 14, 15).

VC and *trans*-DCE are both dechlorinated similarly by *"D. ethenogenes"* strain 195, but because *trans*-DCE is not an intermediate that accumulates to high levels, its role into the overall pathway is of limited importance. Instead, VC plays a fundamental role in the dechlorination of PCE to ETH, where it is the main intermediate accumulated by *"D. ethenogenes"* strain 195. The last step of dechlorination, VC to ETH, is still the limiting reaction to the full reduction of PCE, in accordance with previous results (11, 12). This is in contrast to what it is found in all other studies reported (2, 3, 6, 7, 9, 10, 16, 17). In depth studies regarding VC dechlorination to ETH by *"D. ethenogenes"* strain 195 are described in Chapter Seven.

Experience accrued from more than one hundred transfers of *"D. ethenogenes"* strain 195 with PCE as sole e^- acceptor signaled to the fact that a high first dose of PCE limited the ability of a new transfer to dechlorinate PCE. The gradual inhibition by PCE of the culture's dechlorinating activity at increasingly high concentrations, or the saturation of the dehalogenating enzyme/s responsible for the overall pathway are potential explanations for this phenomenon. The accumulation of much more *cis*-DCE in cultures utilizing TCE as the direct e^- acceptor instead of PCE (Fig. 5.5) could be explained by TCE being present in far higher concentration in Fig. 5.5 than in Fig. 5.3, combined with the fact that TCE is the immediate precursor of *cis*-DCE.

"D. ethenogenes" strain 195 transferred to TCE, 1,1-DCE or DCA as sole e^- acceptors could be successfully transferred again on these substrates, indicating growth on these compounds. After the culture had consumed several doses of PCE, TCE, 1,1-DCE or DCA, *"D. ethenogenes"* strain 195 was able to immediately switch to a different chloroaliphatic with an equal or greater number of chlorines, dechlorinating them all to VC and ETH. The relative rates of chloroethene degradation were different for the same compound depending on the combination of chloroethenes used (Table 5.1; results for *cis*-DCE will be analized in the next chapter). If the new chloroethene had the same number of chlorines than the one the culture had been utilizing for some time, the initial rates of dechlorination of both compounds were similar (i.e. Fig. 5.8). At the same time, newly added substrates with one or two chlorines more than the chloroethene the culture had been utilizing for some time were initially dechlorinated more slowly, with the exception of TCE to PCE. The rate values obtained in the studies performed on DCA (Fig. 5.13) show the same patterns of relative dechlorination rates among chloroethenes as have been described above. A difference with past studies (18) is that a very small but significant amount of VC was accumulated by *"D. ethenogenes"* strain 195.

TABLE 5.1 Relative rates of chloroaliphatic degradation, in percentages (%). After the culture had consumed several doses of a particular chloroaliphatic (shown as "growth substrate"), a different substrate was added to determine the potential for dechlorination of the new compound by the culture (values come from the relative rates obtained in this chapter, except the ones for *cis*-DCE when ustilized as "growth substrate", which come from Chapter Six).

	Relative rates of substrate utilization				
Growth Substrate	PCE	TCE	*cis*-DCE	1,1-DCE	DCA
TCE	133	100	-	-	-
cis-DCE	0	39.1	100	117.4	-
1,1-DCE	78.7	88.7	105	100	-
DCA	78.2	107.7	61.5	61.5	100

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CHAPTER SIX

REDUCTIVE DECHLORINATION OF *cis*-DCE BY *"Dehalococcoides ethenogenes"* strain 195

"I'm not young enough to know everything"

Sir J.M. Barrie 1860-1937
The admirable Crichton (performed 1902, pubd. 1914) act 1

6.1 ABSTRACT.

cis-DCE is a substrate of environmental significance because it often accumulates in contaminated aquifers in which PCE or TCE undergo reductive dechlorination. *"Dehalococcoides ethenogenes"* strain 195 consumed increasing doses of *cis*-DCE at increasing rates over time, indicative of growth on this compound. Doses of *cis*-DCE above 0.2 mmol/l at the time of inoculation (2% v/v inoculum) were toxic to the culture. After growing with *cis*-DCE, *"D. ethenogenes"* strain 195 could utilize TCE, 1,1-DCE or DCA, and these substrates, together with *cis*-DCE, could be freely interchanged as sole electron acceptors, although some combinations were dechlorinated faster than others. After cells had grown with *cis*-DCE, PCE could only be dechlorinated if an alternate substrate, such as 1,1-DCE, was dechlorinated before adding any PCE and after *cis*-DCE had been consumed; or if PCE and *cis*-DCE were fed together at the time of inoculation. Though not completely understood, utilizing *cis*-DCE as the sole electron acceptor effects an inhibitory and/or repressive action on PCE dechlorination.

6.2 INTRODUCTION.

cis-DCE is an environmentally significant product of PCE dechlorination. This is a compound frequently found (accumulated) in contaminated sites (2, 7) and very little is known not only about the dynamics of *cis*-DCE degradation in the environment, and also in the laboratory. As introduced in Chapter Five of this dissertation, several pure cultures have been described that reductively dechlorinate PCE and TCE to mainly *cis*-DCE (3-5, 8-10). There is, then, little known information about how *cis*-DCE is utilized by pure cultures. This knowledge is fundamental to complement an understanding of the processes that lead to *cis*-DCE accumulation in contaminated sites.

In Chapter Five of this dissertation it was determined that *cis*-DCE, although used as growth substrate, was utilized at a slower rate than the other electron (e^-) acceptors. This instigated a deeper look into the dynamics of *cis*-DCE reductive dechlorination by *"D. ethenogenes"* strain 195 when utilized as sole e^- acceptor. This chapter presents the results obtained.

6.3 MATERIALS AND METHODS.

6.3.a Chemicals and analyses of chloroethenes.

PCE, *cis*-DCE, H_2, and other chemicals were purchased and utilized as described in point 2.3.a of Chapter Two.

For quantitative analysis of chloroethenes and ETH, 100-µl, headspace samples were analyzed using a Perkin Elmer Gas Chromatograph 8500, as described in point 3.3.b in Chapter Three of

this dissertation.

6.3.b Growth medium and culture conditions.

The basal salts medium utilized for *"D. ethenogenes"* strain 195 contained the same ingredients as described in Chapter Two of this dissertation. The protocol followed to produce this basal salts medium, previous to the addition of the amendments, was also followed as described in Chapter Two. This medium received the following sterile and anaerobic additions before inoculation (final medium volume ≈ 10 ml/tube): $Na_2S \cdot 9H_2O$, 2 mM (added last); $NaHCO_3$, 12 mM (added first); the ABSS amendments {filter-sterilized anaerobic digestor sludge supernatant (SS; prepared as described in Chapter Two)), 25% v/v; sodium acetate, 2 mM; and vitamin solution (1) concentrated 10-fold, 0.5% v/v}; and 5% v/v extract from the Butyrate/PCE culture (prepared as described in Chapter Three). The antibiotic ampicillin, added at 0.3 μg/ml to the cultures, was prepared as described in Chapter Three.

Unless otherwise stated, inoculum sizes were 2% v/v, all incubations were done in duplicate, and each experiment presented was performed at least twice with similar results. Duplicate tubes performed similarly in most of the experiments presented (less than 5% difference in results for tubes under same conditions). Cultures were incubated upside down in the dark as described in Chapter Two.

6.4 RESULTS.

6.4.a Effect of *cis*-DCE concentration on dechlorination.

Fig. 6.1 (a) and (b) show the formation of products from *cis*-DCE when utilized as sole e^- acceptor by a culture of *"D. ethenogenes"* strain 195. As shown in Fig. 6.1, (a) the organism was able to dechlorinate *cis*-DCE added at standard amounts for PCE dechlorination (0.3, 0.5 and 0.7 mmol/l *cis*-DCE) almost exclusively to VC. Nevertheless, dechlorination rates, in accordance with similar results obtained in Fig. 5.1, were slower than the ones obtained previously from PCE, TCE or 1,1-DCE (Chapter Five). The culture dechlorinated the 0.5 mmol/l dose of *cis*-DCE faster than the first, but a third dose of 0.7 mmol/l *cis*-DCE was not completely dechlorinated even after 8.5 days. It was observed in subsequent experiments (data not shown) that *cis*-DCE concentrations above 0.5-0.6 mmol/l were inhibitory to the culture.

When the initial amount of *cis*-DCE added as sole e^- acceptor to the same culture was reduced by 50% to ca. 0.15 mmol/l {Fig. 6.1 (b)}, this initial dose was dechlorinated 15% faster, as measured by VC accumulation, than the data in Fig. 6.1 (a) predicted. The overall performance of the culture was also better; after 8.5 days from the start of the experiments, the culture in Fig. 6.1 (b) produced a total of 0.92 mmol/l VC against the 0.52 mmol/l VC produced by the culture in Fig. 6.1 (a). In both experiments, *cis*-DCE was dechlorinated faster with time, indicative of growth of the culture on this compound. The maximum rate of dechlorination of *cis*-DCE, while being utilized as a growth substrate, was 0.25±0.05 mmol/l.day {corresponding to data from Fig. 6.1 (b)}. ETH was not produced from VC in the presence of

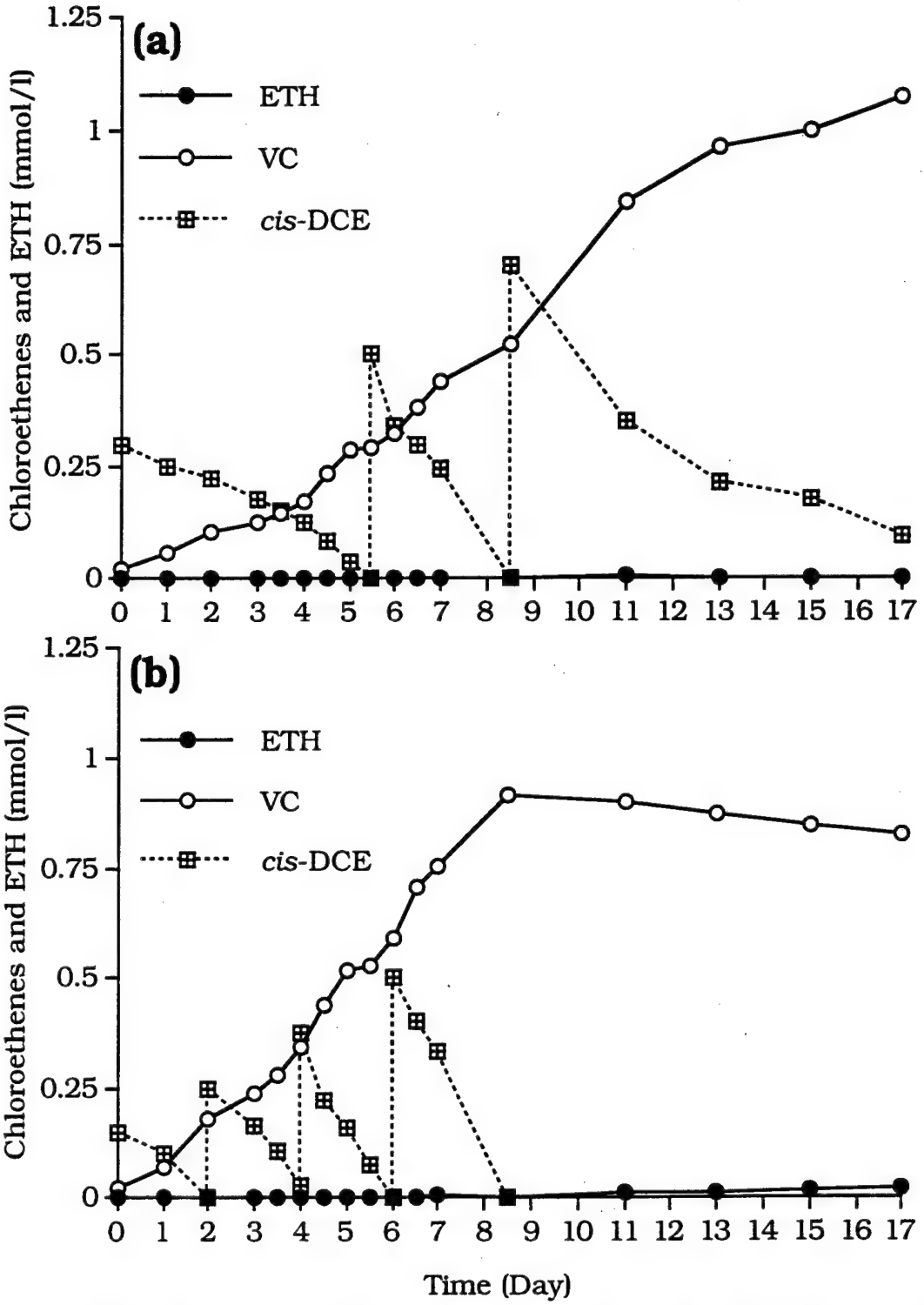


FIGURE 6.1 Effects of different amounts of *cis*-DCE added as sole electron acceptor to cultures transferred into medium with all requirements for growth present. The doses of *cis*-DCE added to the culture were: **(a)** 0.3, 0.5 and 0.7 mmol/l and **(b)** 0.15, 0.25, 0.35 and 0.50 mmol/l.

cis-DCE and, as seen in Fig. 6.1 (b) it was only produced to limited amounts once *cis*-DCE had been depleted. Cultures grown on *cis*-DCE could be successfully transferred on the same substrate indefinitely.

Fig. 6.2 shows the result of feeding *cis*-DCE to a culture that had been allowed to reach maximum cell density (see Fig. 3.8 for reference) while growing on PCE. The amounts of the three *cis*-DCE doses administered to the culture were the same as those fed to the culture in Fig. 6.1 (a) (0.3, 0.5 and 0.7 mmol/l). In this experiment, the three *cis*-DCE doses were dechlorinated in only 2 days, about 10-fold faster than the ones fed to the culture of Fig. 6.1 (a).

6.4.b Substrate interchangeability in cultures grown on *cis*-DCE.

An experiment was designed to test the ability of *"D. ethenogenes"* strain 195 to dechlorinate other chloroethenes of equal or higher number of chlorines after having consumed several doses of *cis*-DCE as sole e^- acceptor. A culture grown on *cis*-DCE was transferred and fed with three consecutive doses of *cis*-DCE (0.15, 0.25 and 0.35 mmol/l), followed by an alternative substrate. Figure 6.3 (a), (b) and (c) show the results of the fourth dose, which was of 0.35 mmol/l of *cis*-DCE, 1,1-DCE or TCE respectively. Fig. 6.3 (d) depicts the complete sequence of feedings from Day 0, showing a fourth feeding of 0.35 mmol/l PCE. *"D. ethenogenes"* strain 195 was able to dechlorinate *cis*-DCE, 1,1-DCE and TCE without any lag phase. *cis*-DCE and 1,1-DCE were both dechlorinated to VC with very similar rates: 0.23±0.03 mmol l^{-1} day^{-1} for *cis*-DCE and 0.27±0.02 mmol l^{-1} day^{-1} for 1,1-DCE. A little ETH started to be formed from VC as the

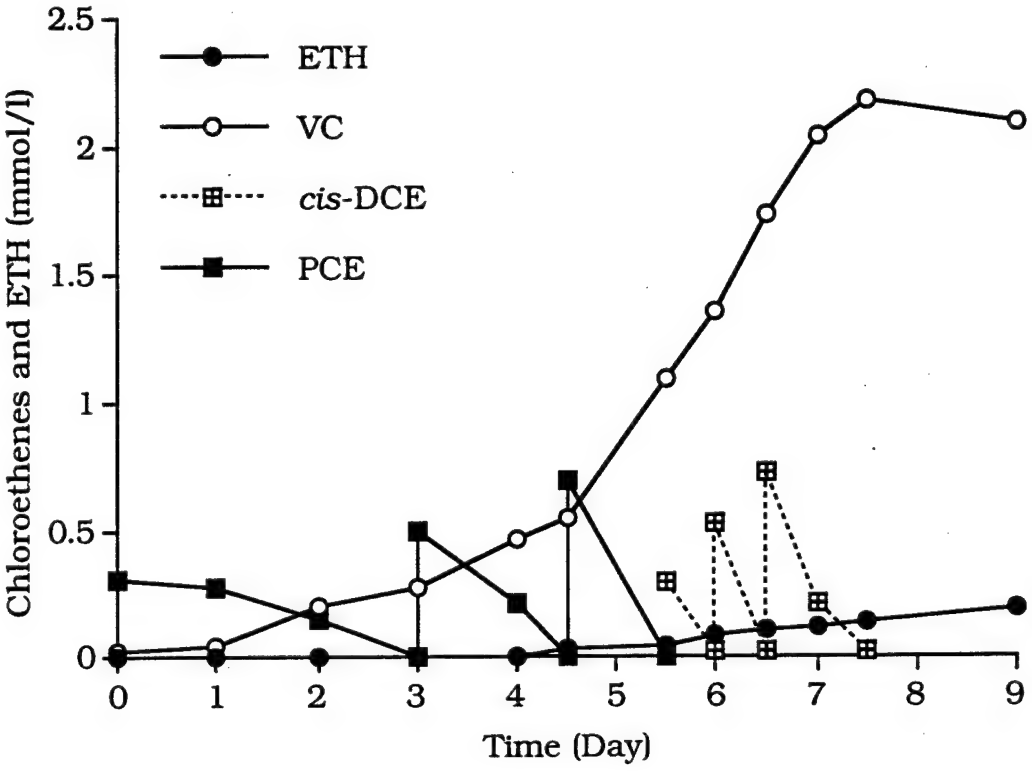
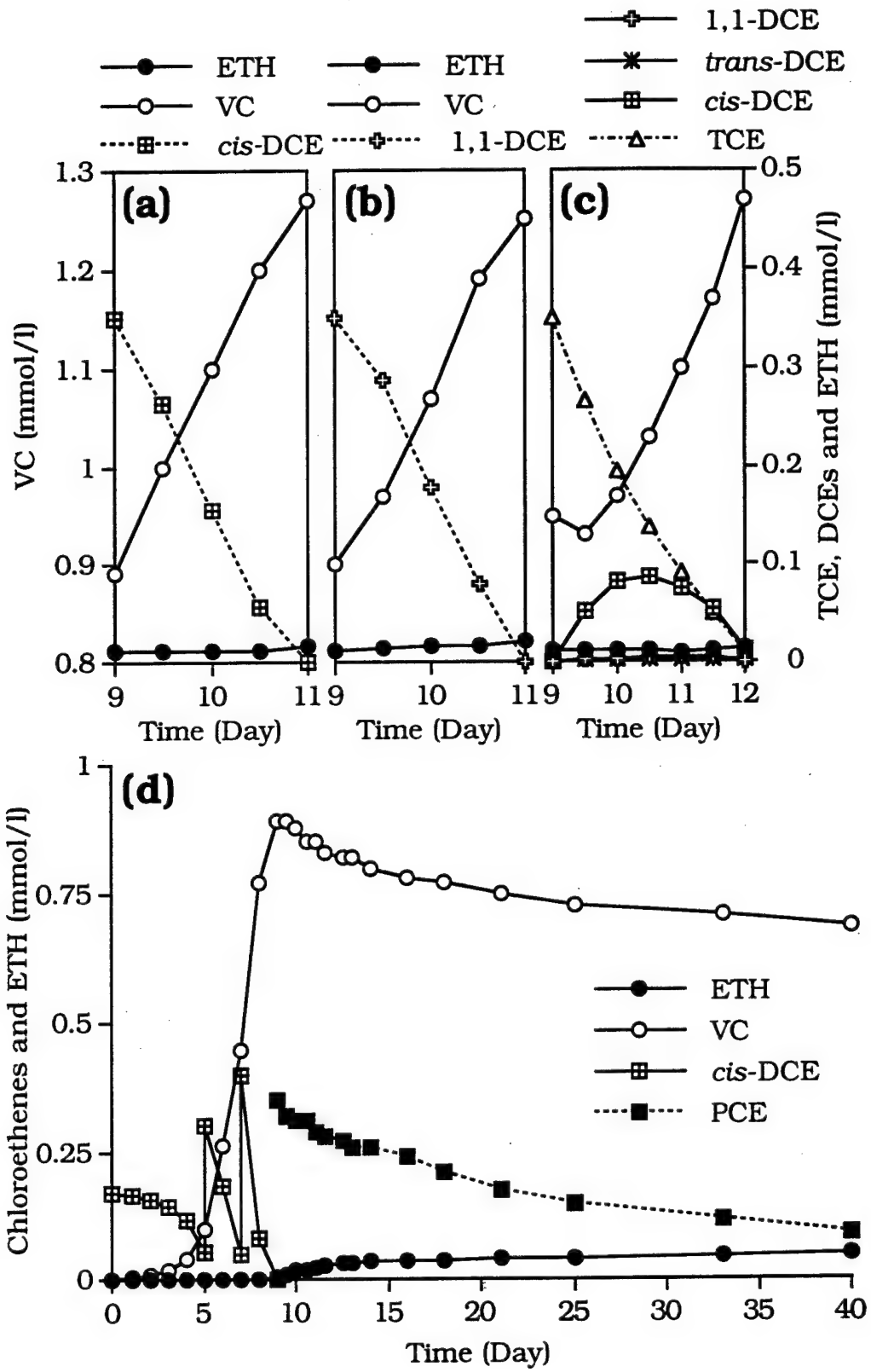


FIGURE 6.2 Effect of adding high amounts of *cis*-DCE (0.3, 0.5 and 0.7 mmol/l) after feeding the culture with three consecutive standard doses of PCE (0.3, 0.5 and 0.7 mmol/l).

FIGURE 6.3 *cis*-DCE **(a)**, 1,1-DCE **(b)**, TCE **(c)** and PCE **(d)** disappearance and product formation in cultures transferred (2% v/v inoculum) with *cis*-DCE as sole electron acceptor. In all experiments, *cis*-DCE was fed three consecutive times (0.15, 0.25 and 0.35 mmol/l). Graphs **(a)**, **(b)** and **(c)** show the fourth feeding, which was of 0.35 mmol/l of *cis*-DCE, 1,1-DCE and TCE respectively. Graph **(d)** shows the complete sequence of feedings from Day 0, being the fourth feeding 0.35 mmol/l PCE, which was added to the culture at Day 8.


ETH
VC
cis-DCE
1,1-DCE
trans-DCE
TCE
(a)
(b)
(c)
(d)
VC (mmol/l)
TCE, DCEs and ETH (mmol/l)
Time (Day)
Chloroethenes and ETH (mmol/l)
PCE

concentrations of both chloroethenes diminished to almost zero. TCE dechlorination was much slower than that of the DCEs (rate of 0.09±0.02 mmol l^{-1} day^{-1}). TCE was initially dechlorinated to *cis*-DCE (no VC was produced in the first 1/2 day). After 1.5 days of *cis*-DCE accumulation, this chloroethene was gradually dechlorinated together with the remaining TCE, while VC accumulated as the dechlorination product of both. PCE, contrary to the other chloroethenes tested, was not dechlorinated at all for 32 days after it was added to the culture {Fig. 6.3 (d)}. The PCE concentration diminished with time due to its absorption to and transfer through the highly punctured stopper (Fig. 2.3). Moreover, contrary to past observations, ETH was produced from VC in the presence of PCE.

6.4.c PCE utilization by cultures grown on *cis*-DCE.

To investigate further whether this loss of ability to use PCE after growing on *cis*-DCE was irreversible, the same culture utilized as inoculum in the experiment of Fig. 6.3 was inoculated into fresh medium and fed three times with *cis*-DCE (see Fig. 6.4). In this experiment, though, two doses (0.4 ad 0.55 mmol/l) of 1,1-DCE were fed to the culture as an alternative substrate before adding PCE. As shown in Fig. 6.4, the culture was then able to dechlorinate PCE. The dechlorination of the first PCE dose was 4 to 5 days slower than that for a culture accustomed to grow on PCE, TCE or 1,1-DCE. Still, the rate of PCE degradation increased with time with very little buildup of intermediates (data not shown).

The same approach as above was followed when transferring the culture. Fig. 6.5 (a) shows the results of a culture that had been

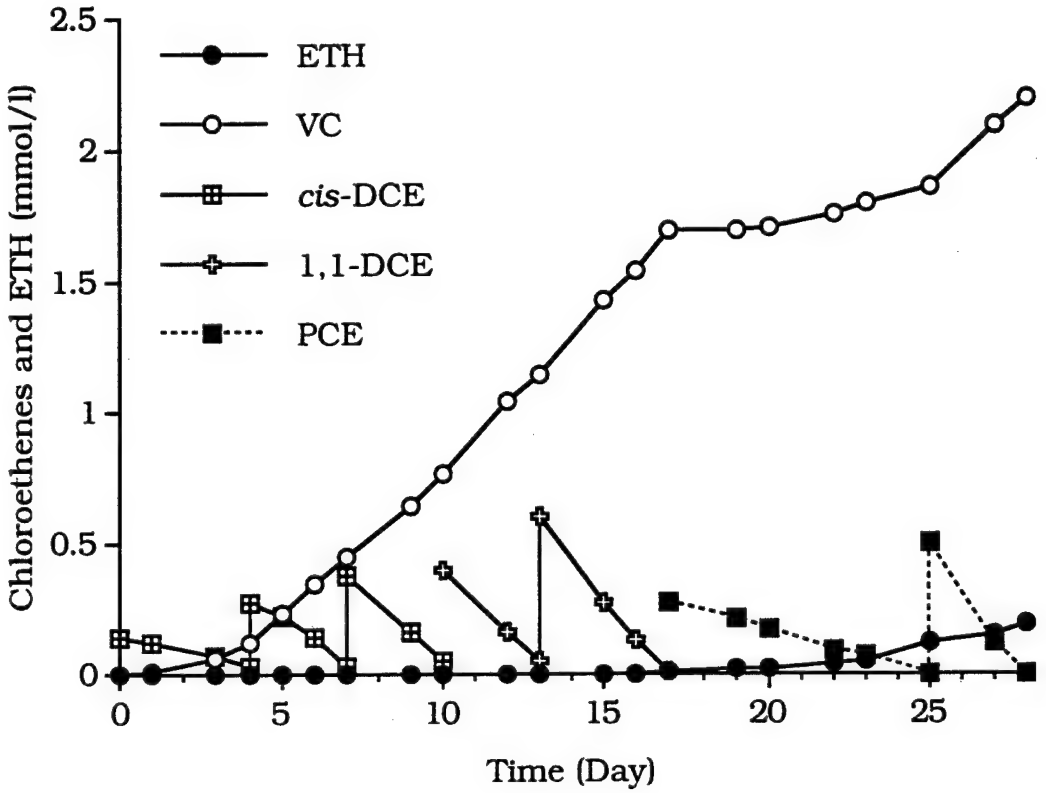


FIGURE 6.4 PCE disappearance and product formation by D. ethenogenes strain 195 grown with all the necessary requirements. Two doses of 1,1-DCE (0.4 and 0.55 mmol/l) were added right after the three initial doses of *cis*-DCE (0.15, 0.25 and 0.35 mmol/l) were consumed.

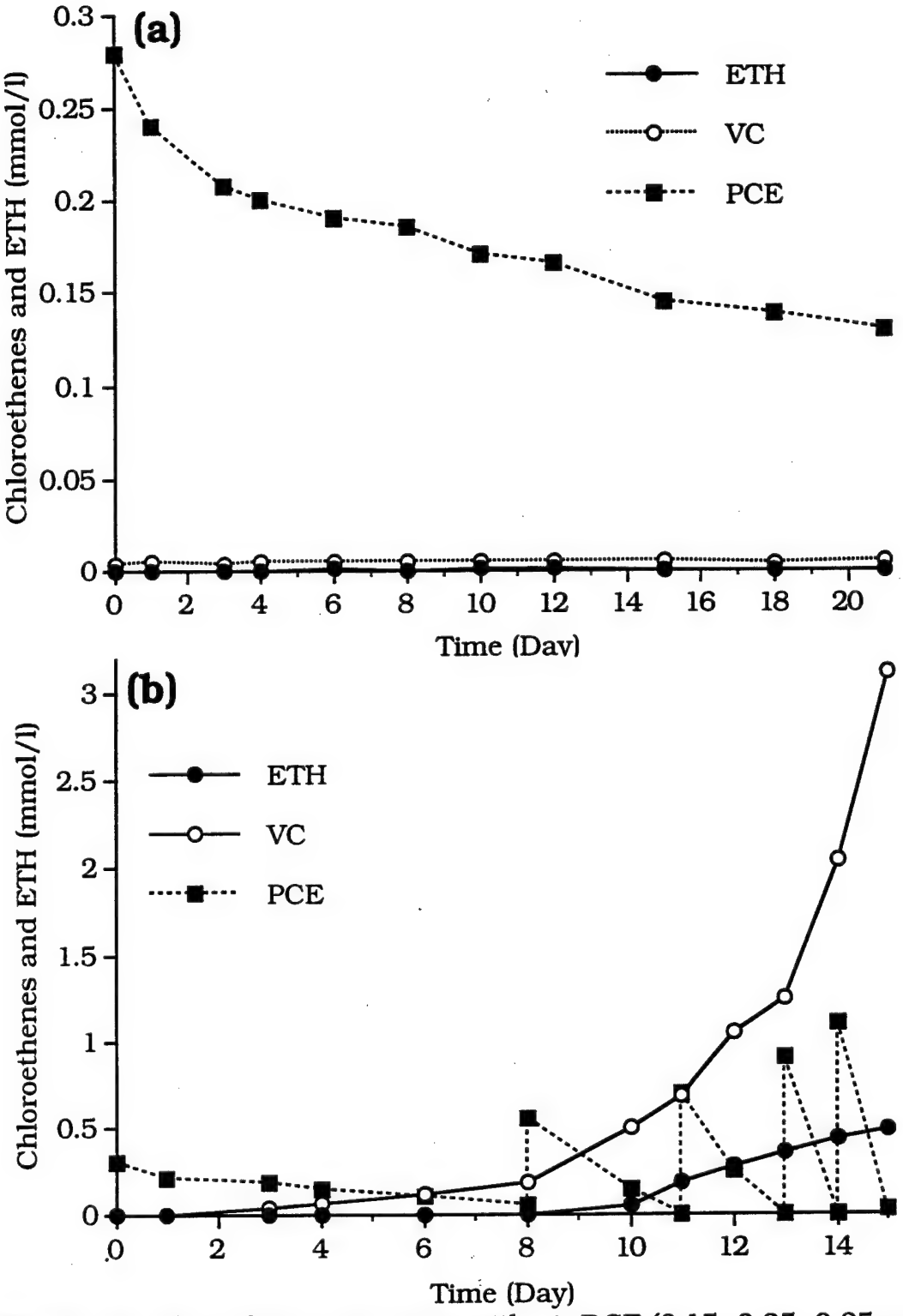


FIGURE 6.5 The culture was grown with *cis*-DCE (0.15, 0.25, 0.35 and 0.45 mmol/l) and then transferred with PCE **(a)** or with 1,1-DCE (not shown). This later culture was transferred a second time with PCE **(b)**.

growing on *cis*-DCE (1.21 mmol/l VC and ETH produced in 17 days) and was transferred into fresh medium containing PCE as sole e^- acceptor. *"D. ethenogenes"* strain 195 did not dechlorinate PCE under these conditions, since no products were detected. The amount of PCE diminished with time due to its absorption into the stopper. In a parallel experiment, the same culture growing on *cis*-DCE was transferred and grown with 1,1-DCE as sole e^- acceptor (1.35 mmol/l VC and ETH produced in 11 days), and then this culture was transferred again into fresh medium containing PCE as sole e^- acceptor. Figure 6.5 (b) shows that PCE was dechlorinated by this culture. Again, the dechlorination of the first PCE dose was 4 to 5 days slower than the regular time a culture accustomed to grow on PCE, TCE or 1,1-DCE would have taken. The rate of PCE degradation increased with time, reaching high rates of degradation by the fourth PCE dose, which indicates normal growth of the organism on this compound.

Fig. 6.6 shows the dechlorination patterns of *cis*-DCE and PCE when added together to a culture that had been growing to high cell density with PCE as sole e^- acceptor. About 0.35 mmol/l of PCE and *cis*-DCE were added to the culture on Day 7. Both compounds were degraded at the same time and at very similar rates.

Figs. 6.7 and 6.8 show the dechlorination patterns of cultures to which both PCE and *cis*-DCE were added at the time of inoculation. The inoculum utilized for the experiment of Fig. 6.7 was grown with PCE as sole e^- acceptor. Both PCE and *cis*-DCE were dechlorinated to VC and ETH. *cis*-DCE was dechlorinated before PCE, and PCE dechlorination was delayed but started before *cis*-DCE had been

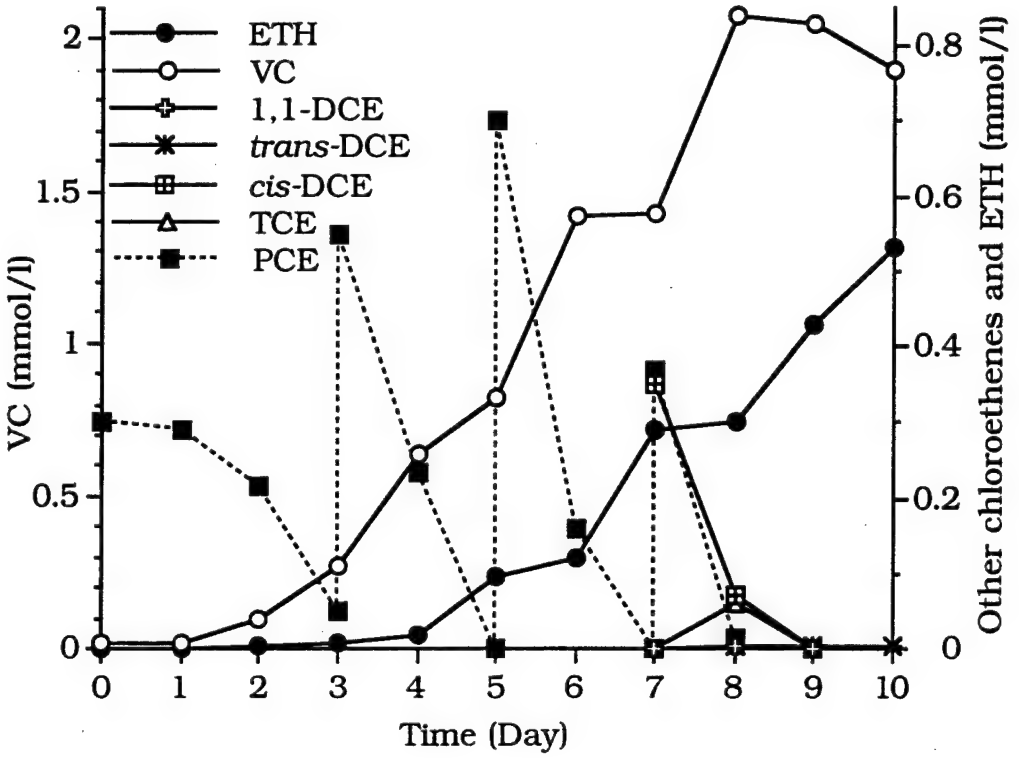


FIGURE 6.6 Reductive dechlorination of PCE and *cis*-DCE when fed together to a culture after three successive doses of PCE. Note that the scale for VC is separate from the rest and it is located on the left Y axis.

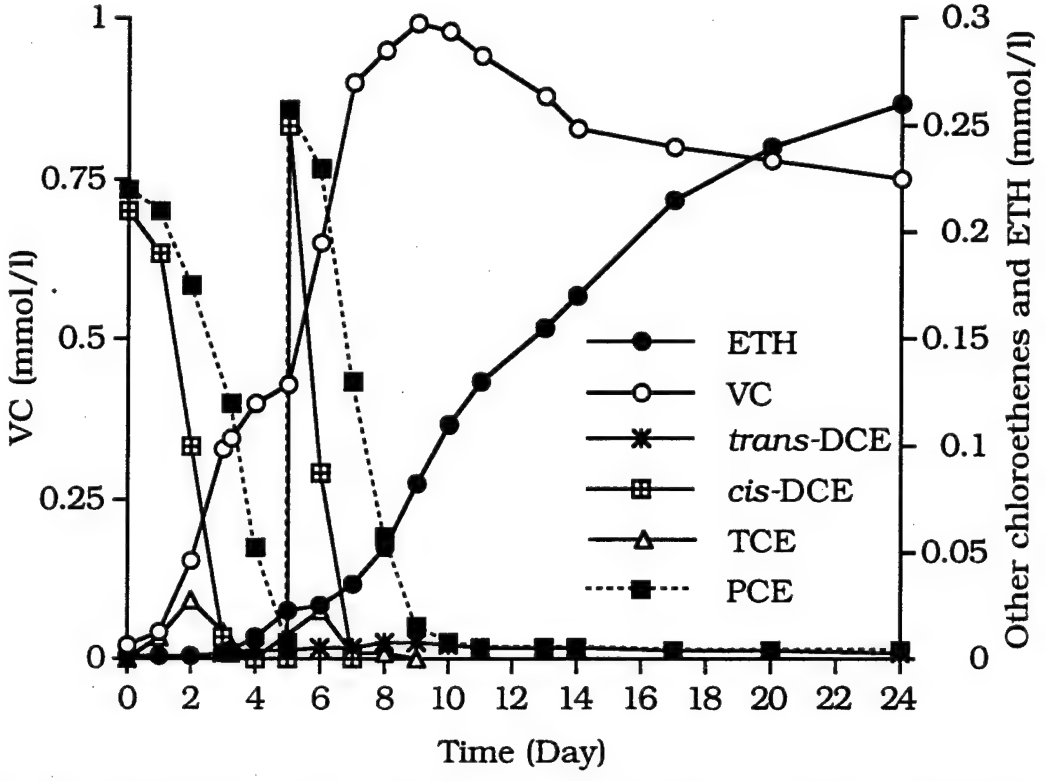


FIGURE 6.7 PCE and *cis*-DCE dechlorination patterns when these compounds were added together at the time of inoculation. The inoculum used for this experiment was grown on PCE.

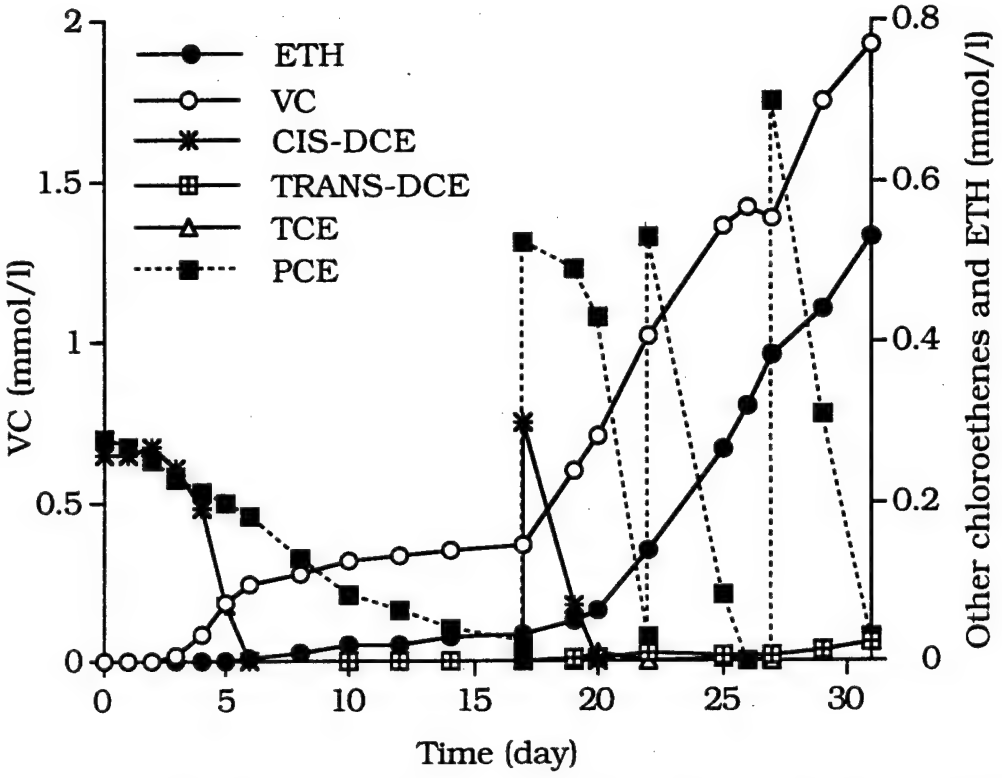


FIGURE 6.8 PCE and *cis*-DCE dechlorination patterns when these compounds were added together at the time of inoculation. The inoculum used for this experiment was grown on *cis*-DCE.

consumed. A small amount of PCE remained for at least 14 days after the second dose of PCE had been mostly consumed, with ETH being formed during that period. TCE and a little *trans*-DCE accumulated during both PCE feedings for the first two and one days respectively and were dechlorinated in both cases before PCE had been consumed (about the time *cis*-DCE levels reached zero). ETH was produced in the presence of PCE, as in Fig. 6.3 (d).

The inoculum utilized for the experiment shown in Fig. 6.8 was grown with *cis*-DCE (about 1 mmol/l consumed) as sole e^- acceptor instead of PCE. As seen in Fig. 6.7, both PCE and *cis*-DCE were degraded to VC and ETH. *cis*-DCE was dechlorinated before PCE started being degraded in contrast with the experiment presented in Fig. 6.7. PCE was dechlorinated to a low extent during the first 17 days with very little VC and ETH production. After the second *cis*-DCE dose had been consumed, PCE was dechlorinated faster with time, indicative of growth on this compound. Two subsequent doses of PCE were dechlorinated in the absence of added *cis*-DCE. Small amounts of *trans*-DCE accumulated constantly during the time the experiment was allowed to run. As shown in Fig. 6.7, ETH accumulated during PCE dechlorination.

6.5 DISCUSSION.

The study of cell growth and dechlorination on *cis*-DCE by this bacterial culture is specially important due to the frequently found accumulation of this compound in sites contaminated with PCE and TCE (2, 7), where *cis*-DCE is often the prevalent chloroethene. The

results obtained here increase our knowledge regarding the reductive dechlorination of *cis*-DCE, since all organisms isolated so far (3-5, 9, 10), with the exception of *"D. ethenogenes"* strain 195, are not capable of degrading *cis*-DCE. A better understanding of the organisms and pathways responsible for its degradation has direct implications in the *in situ* bioremediation of contaminated sites.

cis-DCE was used relatively slowly as sole e^- acceptor by *"D. ethenogenes"* strain 195 {Fig. 6.1 (a)} compared to PCE, TCE and 1,1-DCE by the same bacterium at the same concentrations (Chapter Five). This finding, though different from, are not comparable to results previously obtained (11) using pre-grown mixed cultures containing *"D. ethenogenes"* strain 195. In these cultures the dechlorination of *cis*-DCE was more easily performed by an excess of cell population than when a culture had to grow with *cis*-DCE as the sole e^- acceptor source from the time of inoculation. Results consistent with the ones obtained by Tandoi *et al.* were also obtained in Figs. 6.2, 5.7 and 5.11 of this dissertation, in which the experiments were also performed with pre-grown cultures.

The slow metabolism of high concentrations of *cis*-DCE by *"D. ethenogenes"* strain 195 {Figs. 6.1 (a) and 5.1} in cultures inoculated into medium with *cis*-DCE as sole e^- acceptor, when compared with the much faster dechlorination rates obtained at lower concentrations {Fig. 6.1 (b)}, points towards a toxic effect of *cis*-DCE on cells. This effect on the culture at the time of inoculation is very similar to that found for PCE (Chapter Three), in which if too much compound is added, the ability of the culture to dechlorinate it diminishes with increasing amounts amended. A difference from cultures fed with PCE

is that a lower amount of *cis*-DCE is needed to produce a similar toxic effect as a first dose on a culture that has been just transferred (2% v/v inoculum): more than 0.23 mmol/l of aqueous PCE concentration (0.4 mmol/l PCE added) in comparison to more than 0.17 mmol/l of aqueous *cis*-DCE concentration (0.2 mmol/l *cis*-DCE added). Also, as Fig 6.1 shows, ETH was not produced from VC in the presence of *cis*-DCE, indicating an inhibitory effect of *cis*-DCE over VC dechlorination to ETH very similar to that produced by PCE and other chloroethenes (see Chapters Three, Five and Seven).

The reversible inability of *"D. ethenogenes"* strain 195 to dechlorinate PCE (and to grow with this compound) after having been grown with *cis*-DCE as sole e^- acceptor is surprising (Figs. 6.3 and 6.5). After growing with *cis*-DCE, *"D. ethenogenes"* strain 195 was able to utilize PCE as sole e^- acceptor in only two cases: (I) when an alternative substrate (such as 1,1-DCE) was dechlorinated before adding PCE (after *cis*-DCE had been consumed); and (II) when both compounds were fed together at the time of inoculation (Fig. 6.8).

Studies on the dehalogenases of mixed cultures containing *"D. ethenogenes"* strain 195, currently being performed (6), show the presence of two enzymes, one active in the reductive dechlorination of PCE to TCE, and another one responsible for the rest of the pathway. If this is true, growth with *cis*-DCE alone could (reversibly) repress the transcription of the gene responsible for the production of the PCE dehalogenase (Fig. 6.8).

Fig. 6.6 shows that there is no inhibition of the PCE dehalogensae by *cis*-DCE, even though some TCE and DCE isomers were produced from PCE by the culture. Fig. 6.7 shows that, when

PCE and *cis*-DCE were added together at the time of inoculation in cultures grown with PCE, PCE dechlorination is delayed and mainly occurs after *cis*-DCE has been consumed (also seen in Fig. 6.8), which could be produced by competition for electrons.

Addition of an alternate substrate (1,1-DCE; Fig. 5.8) after *cis*-DCE consumption and before adding PCE to the culture allowed cells to use PCE as a growth substrate again. Under these conditions, the first PCE dose was always consumed more slowly than usual {Figs. 6.4 and 6.5 (b)}, indicating initial partial recovery of the ability to dechlorinate PCE by *"D. ethenogenes"* strain 195. After a few doses had been consumed, the rates of PCE dechlorination became as fast as usual and the recovery seemed complete. There is no definite explanation for this phenomenon at this point. The results presented in this chapter have been used to develop a possible explanation:

After growing with *cis*-DCE only, no PCE dehalogenase would be present in the cells and not enough energy would be generated to produce any. Under these conditions *"D. ethenogenes"* strain 195 could not dechlorinate or grow with PCE when added alone {seen in Figs. 6.3 and 6.5 (a)}. But, if under the same conditions PCE was added together with a suitable growth substrate, the dechlorination of the additional growth substrate would create enough energy to produce the PCE dehalogenase, which would then be able to start dechlorinating PCE (Fig. 6.8). PCE dechlorination would be slow at the beginning and faster with time until there is enough enzyme that the energy produced from PCE dechlorination is sufficient to synthesize more PCE dehalogenase, making the presence of the other substrate unnecessary {see Fig. 6.8; also seen in Figs. 6.4 and 6.5 (b)}.

Cultures growing on PCE instead of *cis*-DCE would have both PCE and TCE dehalogenases present in the cells and, therefore, would have no trouble dechlorinating PCE and *cis*-DCE when added together, also because these compounds would be reduced by different enzymes (see Fig. 6.6) (6).

It is interesting that in Fig. 6.3 (d), contrary to all past observations, ETH was produced from VC in the presence of PCE. It is therefore not just the presence of PCE that inhibits ETH formation. These results indicate that PCE has to be utilized by the cells to produce the inhibitory effect on ETH formation. At the same time, and in apparent contradiction, ETH was produced while PCE is being dechlorinated in Figs. 6.7 and 6.8. This could indicate competition for electrons or the inability for *"D. ethenogenes"* strain 195 to utilize PCE as sole e^- acceptor after these cells have grown on *cis*-DCE. In these experiments, PCE could have been dechlorinated cometabolically.

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CHAPTER SEVEN

VC DECHLORINATION TO ETH BY "*Dehalococcoides ethenogenes*" strain 195

"If the problem has a solution, why are you worried?
If it doesn't, why are you worried?"

Popular phrase (oriental)

7.1 ABSTRACT.

The last step of dechlorination, VC to ETH, is the rate-limiting step for the complete dechlorination of PCE by *"Dehalococcoides ethenogenes"* strain 195, and the patterns of utilization of VC were typical of a cometabolic process. VC was not utilized as a growth substrate by *"D. ethenogenes"* strain 195. When added as sole electron acceptor, VC was metabolized very slowly to ETH at a constant rate. The rates of VC dechlorination were much greater if a suitable growth substrate like PCE was added, although significant reduction of VC to ETH only occurred after the PCE dose had been consumed. TCE, *cis*-DCE and 1,1-DCE also partially inhibited ETH formation when present in the medium. The rates of ETH production from VC decreased with time in the absence of PCE. When PCE was added to the medium with hexadecane (HDC), a low (0.3 mmol/l), constant amount of PCE in the sample allowed for continuous and increasing ETH production from VC as the culture grew on PCE. Therefore, PCE dechlorination and ETH production can coexist.

7.2 INTRODUCTION.

Although vinyl chloride (VC) has powerful carcinogenic effects on animals (including humans), it is also one of the largest commodity chemicals on which we depend today, used mainly in the production of plastics like PVC. The world-wide production of VC estimated for 1982 was of 17 million tons (17). Drastic reductions of VC production have been effected in many countries since then, although it is still a contaminant commonly found in the environment. Since VC is a relatively reduced molecule, its further reduction to ETH is slow under reducing conditions (7, 9, 12) and, even though it is a very thermodynamically favorable as sole electron (e^-) acceptor (27), VC is often observed to accumulate in anaerobic environments contaminated with PCE or TCE (4, 5, 28).

There is little information available about dechlorination of VC by mixed or pure anaerobic cultures in the laboratory or *in situ*. Anaerobic VC dechlorination has been described in mixed enrichment cultures (7, 9, 21, 24, 25) and in microcosms and *in situ* studies (3-5, 19). Aerobic oxidation of VC is more common (6, 14, 26). In part this is due to the reduced nature of the molecule, which makes its dechlorination with oxygen as terminal e^- acceptor very favorable.

In this chapter, a detailed study of the utilization of VC and the production of ETH by *"D. ethenogenes"* strain 195, in the presence and absence of other chloroethenes, is presented.

7.3 MATERIALS AND METHODS.

7.3.a Chemicals and analyses of chloroethenes.

PCE, VC, H_2, and other chemicals were purchased and utilized as described in point 2.3.a in Chapter Two of this dissertation.

For quantitative analysis of chloroethenes and ETH, samples were analyzed using a Perkin Elmer Gas Chromatograph 8500, as described in Chapter Three of this dissertation.

7.3.b Growth medium and culture conditions.

The basal salts medium utilized for *"D. ethenogenes"* strain 195 contained the same ingredients as described in Chapter Two of this dissertation. The protocol followed to produce this basal salts medium, previous to the addition of the amendments, was also followed as described in Chapter Two. This medium received the following sterile and anaerobic additions before inoculation (final medium volume≈10 ml/tube): $Na_2S \cdot 9H_2O$, 2 mM (added last); $NaHCO_3$, 12 mM (added first); the ABSS amendments {SS (prepared as described in Chapter Two)), 25% v/v; sodium acetate, 2 mM; and vitamin solution (2) concentrated 10-fold, 0.5% v/v}; and 5% v/v extract from the Butyrate/PCE culture (prepared as described in Chapter Three). Ampicillin, added at 0.3 μg/ml to the cultures, was prepared as described in Chapter Three.

Solutions of PCE in hexadecane (HDC) were prepared as follows: HDC was purged with a mixture of 70% N_2/30% CO_2 for 15 min. The tubes were then sealed with aluminum-crimp Teflon-coated

butyl rubber stoppers (Wheaton, Millville, N.J.) and were autoclaved at 121°C for 30 min. PCE was then added with sterile Swinnex (Millipore Inc., Woburn, MA) filters, after it had been purged with 70% N_2/30% CO_2 for 5 min. HCO_3^-, H_2 and acetate amended to cultures that contained PCE in HDC were added in amounts according to the amount of PCE consumed by these cultures.

Inoculum sizes were 2% v/v, all incubations were done in duplicate, and each experiment presented was performed at least twice with similar results. Duplicate tubes performed very similarly in most of the experiments presented (less than 5% difference in results for tubes under same conditions). Cultures were incubated as described in Chapter Two of this dissertation.

7.4 RESULTS.

7.4.a VC as growth substrate

The ability of *"D. ethenogenes"* strain 195 to grow on VC was examined. A culture grown with PCE was amended with 0.4 mmol/l VC just after inoculation, in the absence of PCE or any other e^- acceptor, as presented in Fig. 7.1 (a). After 30 days of incubation with VC alone, there was only a slow constant conversion of VC to ETH. The slow rate of VC disappearance was attributed to absorption and transfer into and through the stopper as indicated by uninoculated controls (see Fig. 2.3 for reference). A dose of 0.3 mmol/l PCE added at Day 30 was depleted in 14 days with only a slight accumulation of

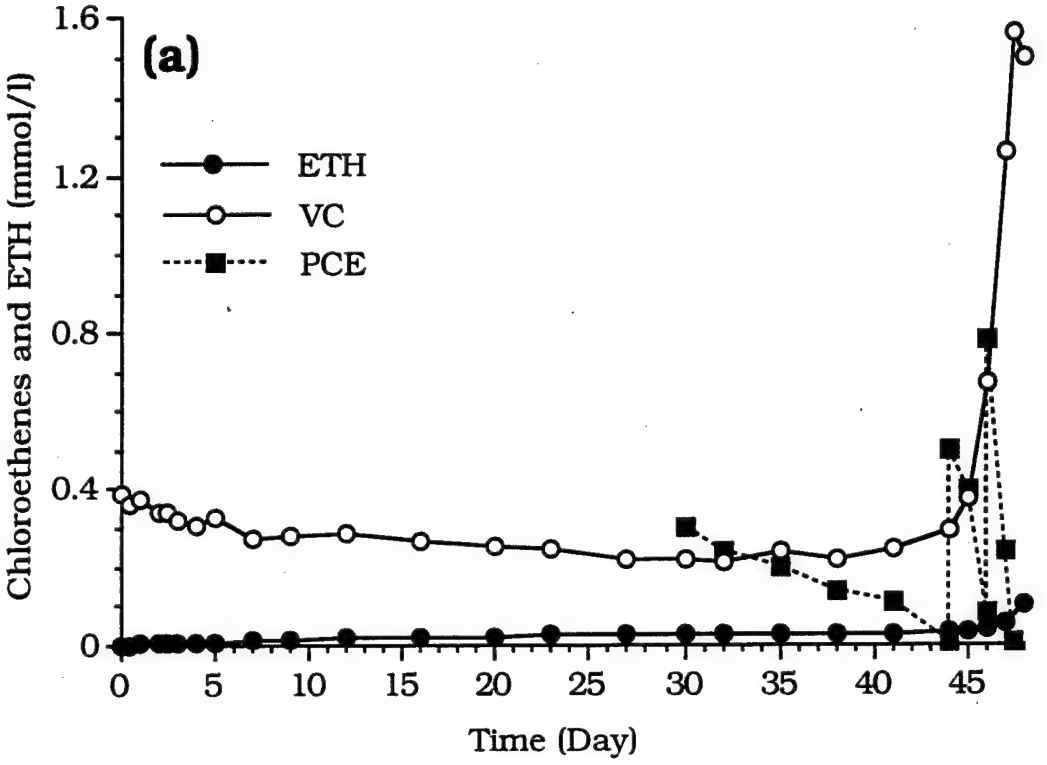
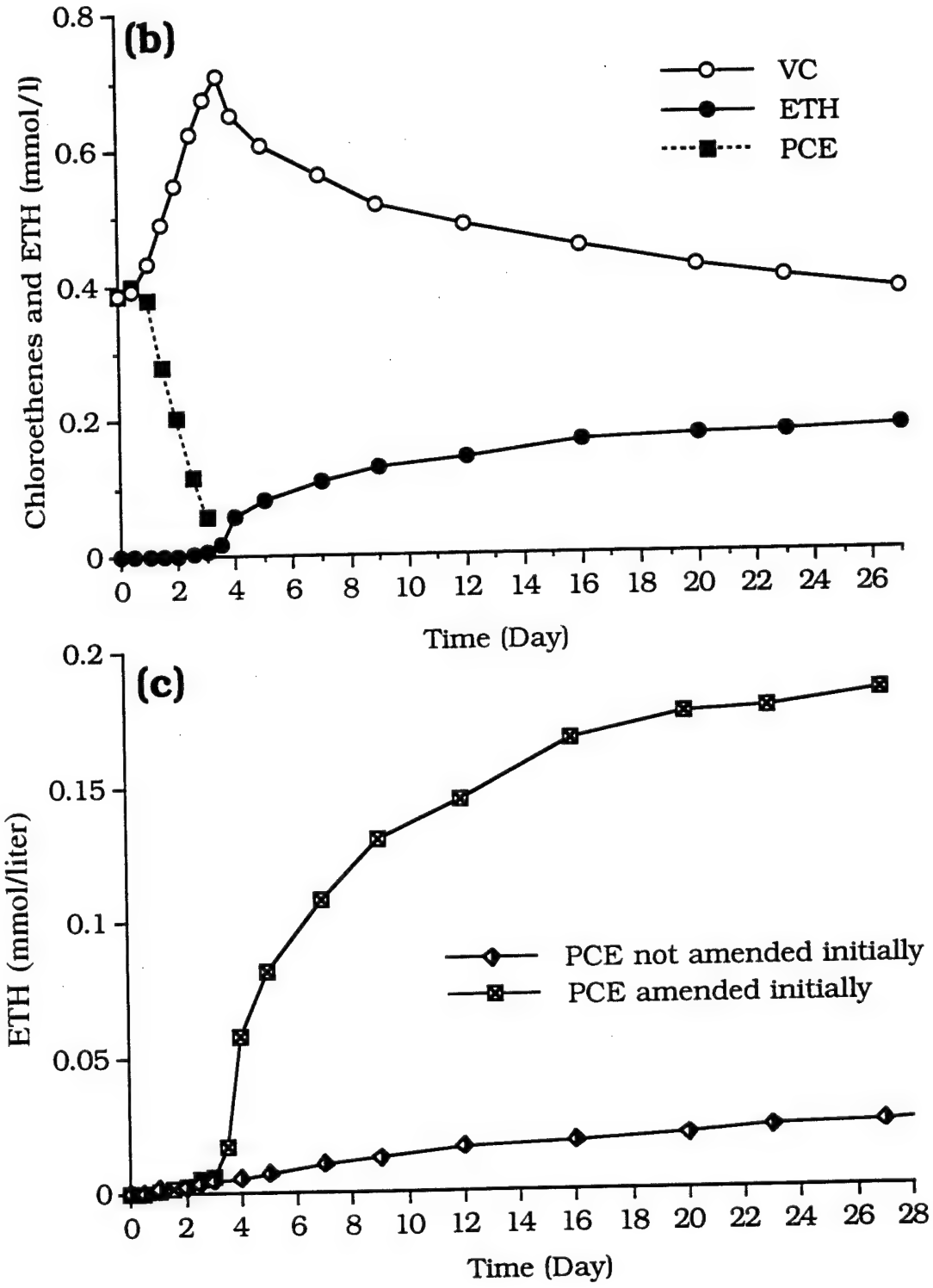


FIGURE 7.1 VC dechlorination to ETH by *"D. ethenogenes"* strain 195 in the absence **(a)** and presence **(b)** of PCE at the time of inoculation. Three consecutively increasing doses of PCE (0.3, 0.5 and 0.7 mmol/l) were added to the culture in **(a)** on days 30, 44 and 46. **(c)** Differences on ETH production by a culture in which PCE was not present for the first 28 days after inoculation {data from **(a)**} and by a culture in which a single dose of PCE (0.4 mmol/l) was added at the time of inoculation {data from **(b)**}.

FIGURE 7.1 Continuation.



dechlorination products detected, with the decrease in PCE concentration attributed mainly to its absorption into the stopper. Two subsequent PCE doses were consumed at increasing rates, with a near stoichiometric conversion to VC and ETH. ETH production ceased in the presence of PCE, from Day 30 to 42, and continued increasing afterwards, mainly in the absence of PCE after the third dose had been consumed and during short periods of low PCE concentration (data not shown).

As shown in Fig. 7.1 (b), when a dose of PCE was added along with VC at Day 0, PCE had been metabolized to VC by Day 4 and, once the PCE was depleted, there was a much more rapid accumulation of ETH than when VC was added in the absence of PCE (at Day 28, the culture that had consumed an initial dose of PCE had produced 0.18 mmol/l ETH against the 0.024 mmol/l produced during the same time by the culture transferred on VC only). Fig. 7.1 (c) shows a close-up comparison of ETH production for the first 28 days of both experiments depicted in Fig. 7.1 (a) and (b). The amount of ETH produced during the first 3 days was the same for both experiments, regardless of PCE being present or absent from the medium. The rates of ETH production from VC by the culture corresponding to Fig. 7.1 (b) decreased with time {see also Fig. 7.1 (b)}, with very little ETH production from Day 28 on (data not shown).

7.4.b ETH production from VC

To demonstrate that ETH production from VC in the absence of PCE was due to bacterial activity, two cultures were transferred into

fresh medium and were amended three consecutive PCE doses (0.2, 0.4 and 0.6 mmol/l), which were consumed during the first six days. At Day 7, one of the cultures was autoclaved (30 min at 121°C). As it can be seen in Fig. 7.2, ETH production completely ceased after autoclaving, while ETH accumulated as expected in cultures that had not been perturbed after the PCE was consumed.

The inhibitory effect of PCE on ETH formation from VC seen in Fig. 7.1 (b) and (c) is examined more closely in Fig. 7.3. ETH formation was measured in relation to the presence or absence of PCE during growth (only PCE and ETH values have been plotted for clarity). Four PCE doses (0.3, 0.5, 0.7 and 0.9 mmol/l) were added to 3 sets of cultures. One of the sets always had PCE present in the medium {Fig. 7.3 (a)}, another did not receive a new PCE dose until 2 days after the previous PCE dose had been consumed {Fig. 7.3 (b)}, and a third one went 4 days without a new PCE dose until the previous PCE dose had been consumed (data not shown). ETH was almost exclusively produced during the periods when PCE was absent from the medium. Fig. 7.3 (c) shows the total amount of ETH produced by the cultures after the four doses of PCE had been consumed by each. Cultures in which PCE was always present produced little ETH (0.014 mmol/l) in contrast to the amount produced by cultures that had had a total of 6 days (0.193 mmol/l ETH) and 12 days (0.25 mmol/l ETH) without PCE.

The remaining chloroethenes that *"D. ethenogenes"* strain 195 is capable of using as e^- acceptors were also tested to determine whether they had a similar inhibitory effect on ETH formation from VC. Fig.

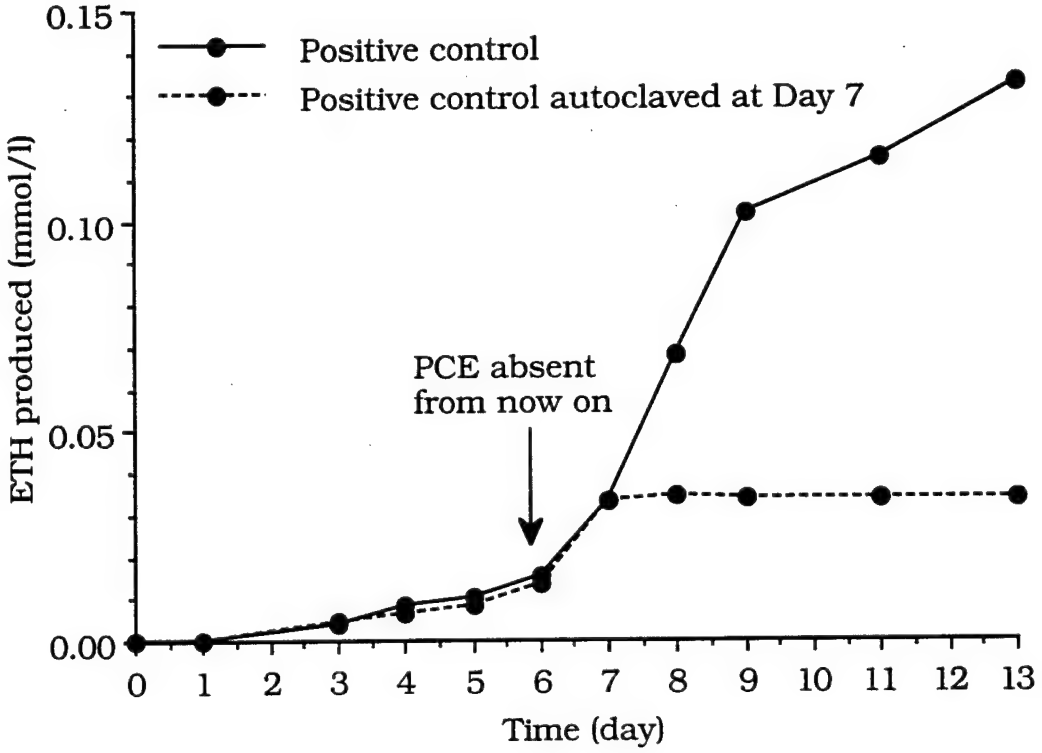
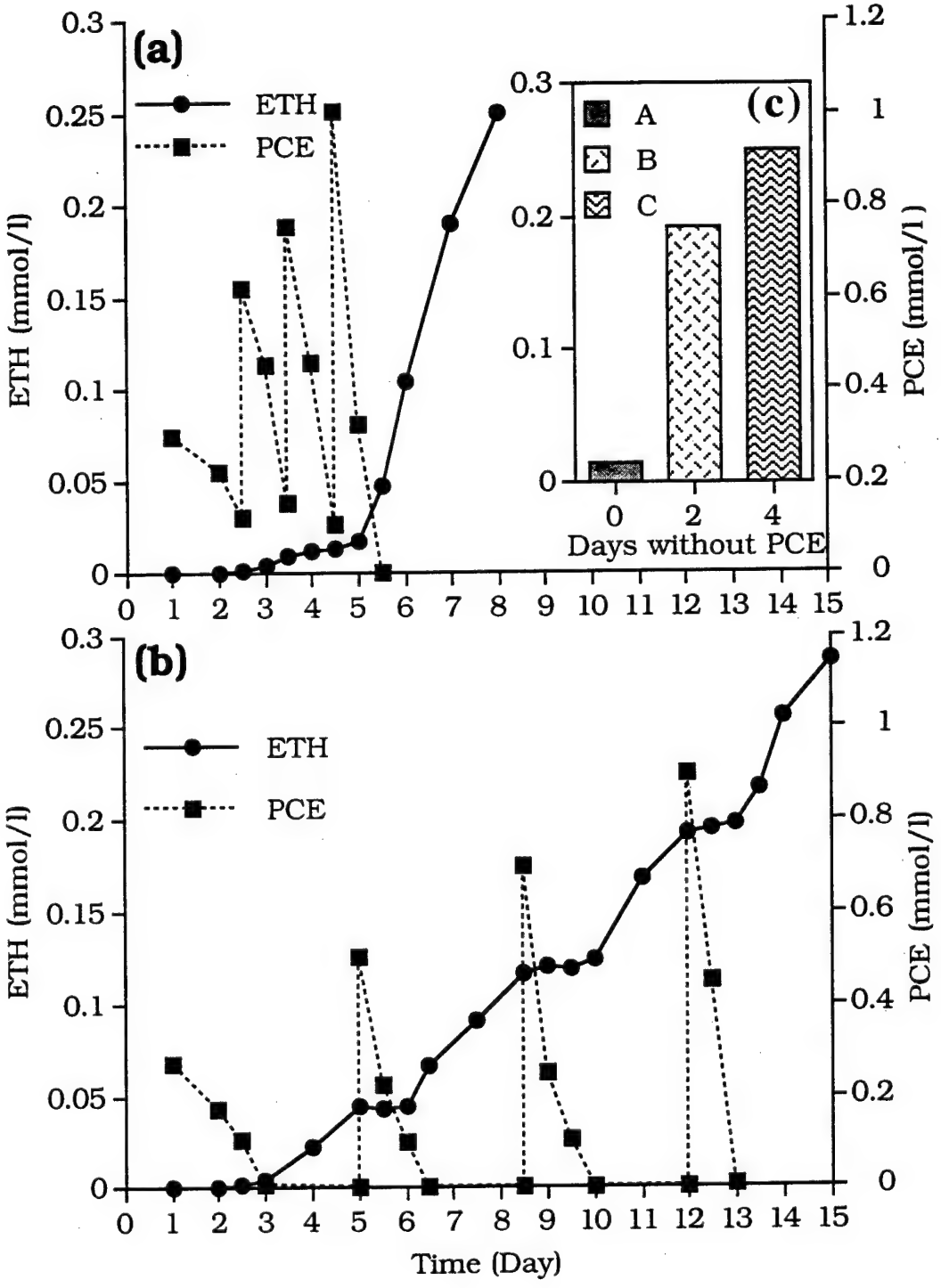


FIGURE 7.2 ETH production by ""*D. ethenogenes*"" strain 195. Both cultures were fed three consecutive doses of PCE (0.2, 0.4 and 0.6 mmol/l). After Day 6, PCE was absent from the medium. At Day 7, one of the cultures (dotted line) was autoclaved (30 min at 121°C).

FIGURE 7.3 ETH formation in relation to the presence or absence of PCE during growth. Cultures were either continuously fed with PCE **(a)** or rested for 2 days after each PCE dose had been consumed **(b)**. **(c)** shows the total amount of ETH produced after 1.44 to 1.5 mmol/l PCE had been consumed in each cultures. A=PCE always present; B=2 days and C=4 days without e^- acceptor between PCE feedings.


(a)
ETH
PCE
ETH (mmol/l)
PCE (mmol/l)
(c)
A
B
C
Days without PCE
(b)
ETH
PCE
ETH (mmol/l)
PCE (mmol/l)
Time (Day)

7.4 shows the results of this experiment. After each culture had consumed two equivalent doses of a particular chloroethene (PCE was consumed in 4 days, TCE in 3.5 days, 1,1-DCE in 3 days and *cis*-DCE in 11 days), the amount of ETH produced in the next two days was measured and compared to that produced in the days before. In all cases, the amounts of ETH produced increased at least 10-fold when the chloroethenes used as growth substrates were absent.

7.4.c Hexadecane (HDC) studies.

The production of ETH was further studied in cultures in which PCE was added, as sole e^- acceptor, in a HDC solution. The HDC created a separate organic phase with high affinity for PCE into which this compound could be concentrated and slowly released into the medium as it was being consumed by the culture. HDC allowed, therefore, the addition of high amounts of PCE while keeping the aqueous concentration of this compound low (15). Fig. 7.5 (a) and (b) shows a comparison for the total amounts of VC and ETH produced by three cultures to which the following amounts of PCE had been added, separately: a total of 14.7 mmol PCE/l added with HDC (about 0.3 mmol/l PCE in aqueous concentration; "low" PCE); 36.7 mmol PCE/l also added with HDC (about 0.6 mmol/l PCE in aqueous concentration; "high" PCE); and 2.4 mmol PCE/l (added in four doses, without HDC; each new dose added before the previous dose had been totally consumed). The HDC solution was added in amounts of 0.75 ml to 9.25 ml of medium, and it did not show any signs of toxicity. Essentially no ETH was dissolved in the HDC phase. As the

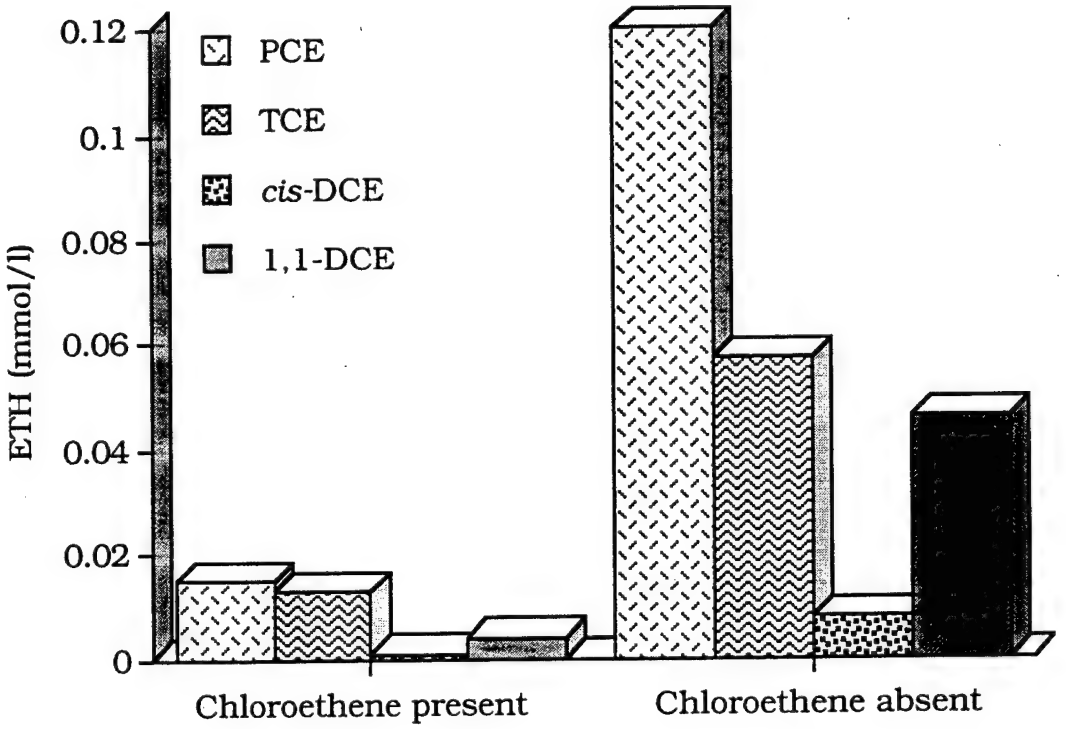


FIGURE 7.4 ETH production from VC during the presence and absence of PCE, TCE, *cis*-DC and 1,1-DCE from the growth medium. Five sets of duplicate tubes consumed, each, two doses of a different chloroethene (0.3 and 0.5 mmol/l). After the respective chloroethenes had been consumed, the amount of ETH produced in the next two days was measured and compared to that produced in the days before.

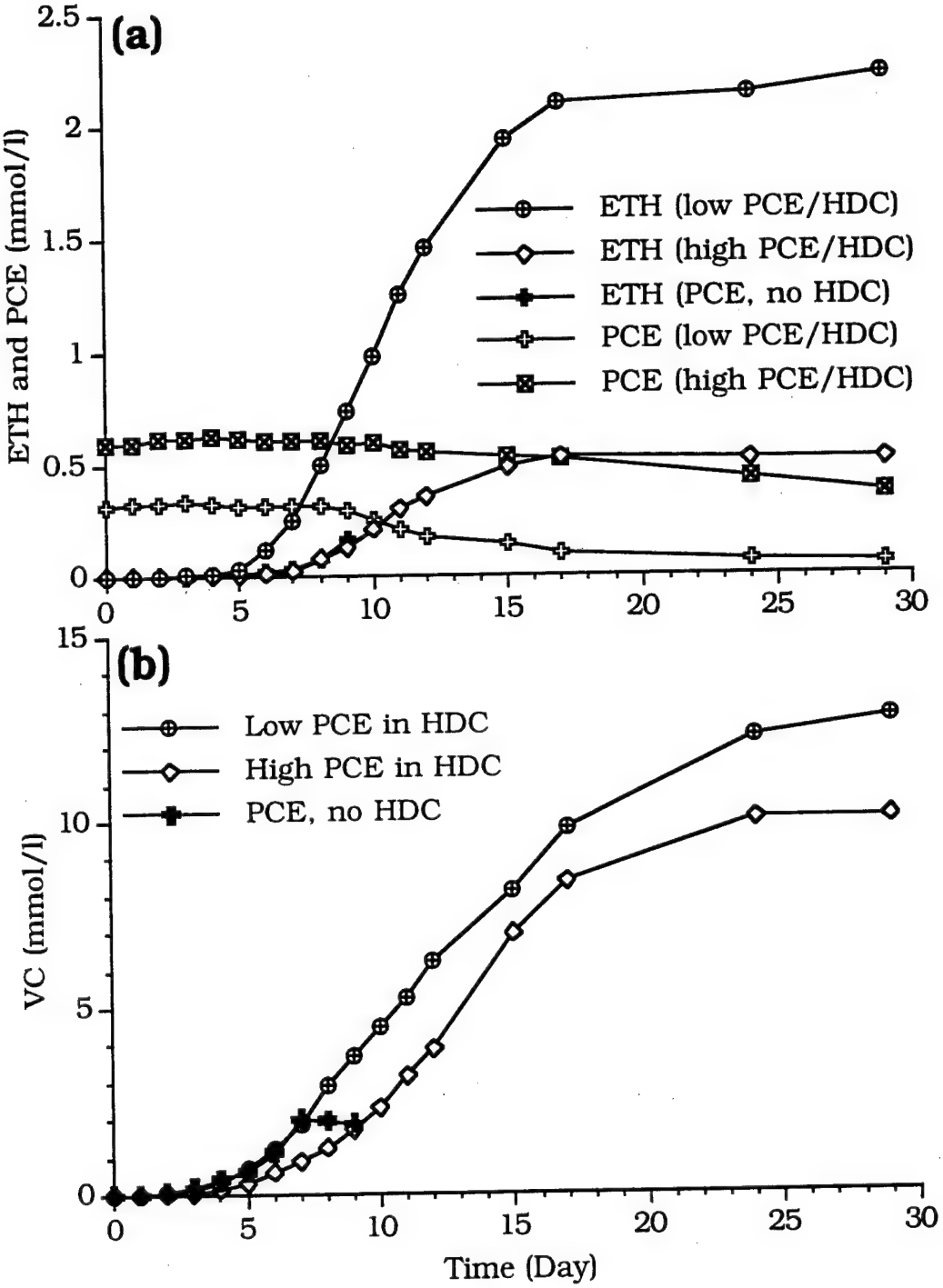


FIGURE 7.5 Total ETH **(a)** and VC **(b)** produced and aqueous levels of PCE **(a)** by 3 transfers of *"D. ethenogenes"* strain 195. PCE was added differently to each transfer: in a HDC phase at "low" (total of 14.7 mmol/l PCE from 0.75 ml of a 2% PCE/HDC solution) and "high" (total of 36.7 mmol/l PCE from 0.75 ml of a 5% PCE/HDC solution) amounts, and without HDC (4 doses; total of 2.4 mmol/l PCE).

degree of chlorination of ethenes increased, there was also an increase in the amount of compound dissolved into the HDC phase (data not shown).

As shown in Fig. 7.5, cultures receiving the "low" PCE/HDC solution produced the same amount of VC as controls without HDC (during the first 7 days) and significantly more VC than cultures amended with the "high" PCE/HDC solution (12.3 against 10 mmol/l at Day 29, respectively). The rates of VC production from PCE by *"D. ethenogenes"* strain 195 slowed down considerably after Day 20. Cultures receiving the "low" PCE/HDC solution produced about 4 fold more ETH than the cultures containing the "high" PCE/HDC solution, which produced the same small amount of ETH during the first 8 days. The rates of ETH production diminished to almost zero after Day 17, a little earlier than the rates for VC production. The amounts of ETH produced by cultures containing the "low" and the "high" PCE/HDC solutions were 2.14 and 0.52 mmol/l, respectively. Both cultures grown with the PCE/HDC solution accumulated TCE and DCE isomers to low levels. The culture amended with the "high" PCE/HDC solution accumulated higher concentrations of intermediates than did cultures amended with the "low" PCE/HDC solution, in which the accumulation of TCE and DCE isomers was small and, at times, negligible (data not shown). DCE isomers normally accumulated to higher amounts than TCE in both cultures (i.e., at Day 13 there were 0.22 mmol/l DCEs and 0.09 mmol/l TCE). Cells viewed by phase-contrast microscopy appeared phase-dark, indicating a healthy state of the cultures (data not shown).

Fig. 7.6 (a) and (b) depicts close ups of Fig. 7.5 (a) and (b), respectively, showing ETH and VC production from PCE during the first 11 days of the experiment (the amounts are shown in aqueous concentrations). In both graphs, ETH production seems to increase about the time when the VC aqueous concentration nearly equal to the constant PCE aqueous concentration present {at about 4.5 days for graph (a) and 7.5 days for graph (b)}.

7.5 DISCUSSION.

Due to the toxicity and cancer producing capabilities of VC (11), combined with the fact that this is a compound that is accumulated by *"D. ethenogenes"* strain 195 as a product of the dechlorination of PCE, it is important to understand the dynamics of its dechlorination.

Results from Fig 7.1 clearly show that, under the present growth conditions, VC cannot be utilized by *"D. ethenogenes"* strain 195 as a growth substrate. VC was metabolized to very little ETH at a constant rate, typical of a cometabolic process. It is interesting that, even though VC is a thermodynamically very favorable e^- acceptor (27), it is not utilized as such by *"D. ethenogenes"* strain 195. The results in Fig. 7.1 (b) indicated the ability of *"D. ethenogenes"* strain 195 to dechlorinate VC faster, once a suitable substrate like PCE was added as e^- acceptor for the culture. These results are similar to those found for *trans*-DCE in Fig. 5.9. PCE serves as an e^- acceptor for energy conservation coupled to growth, thereby building up sufficient

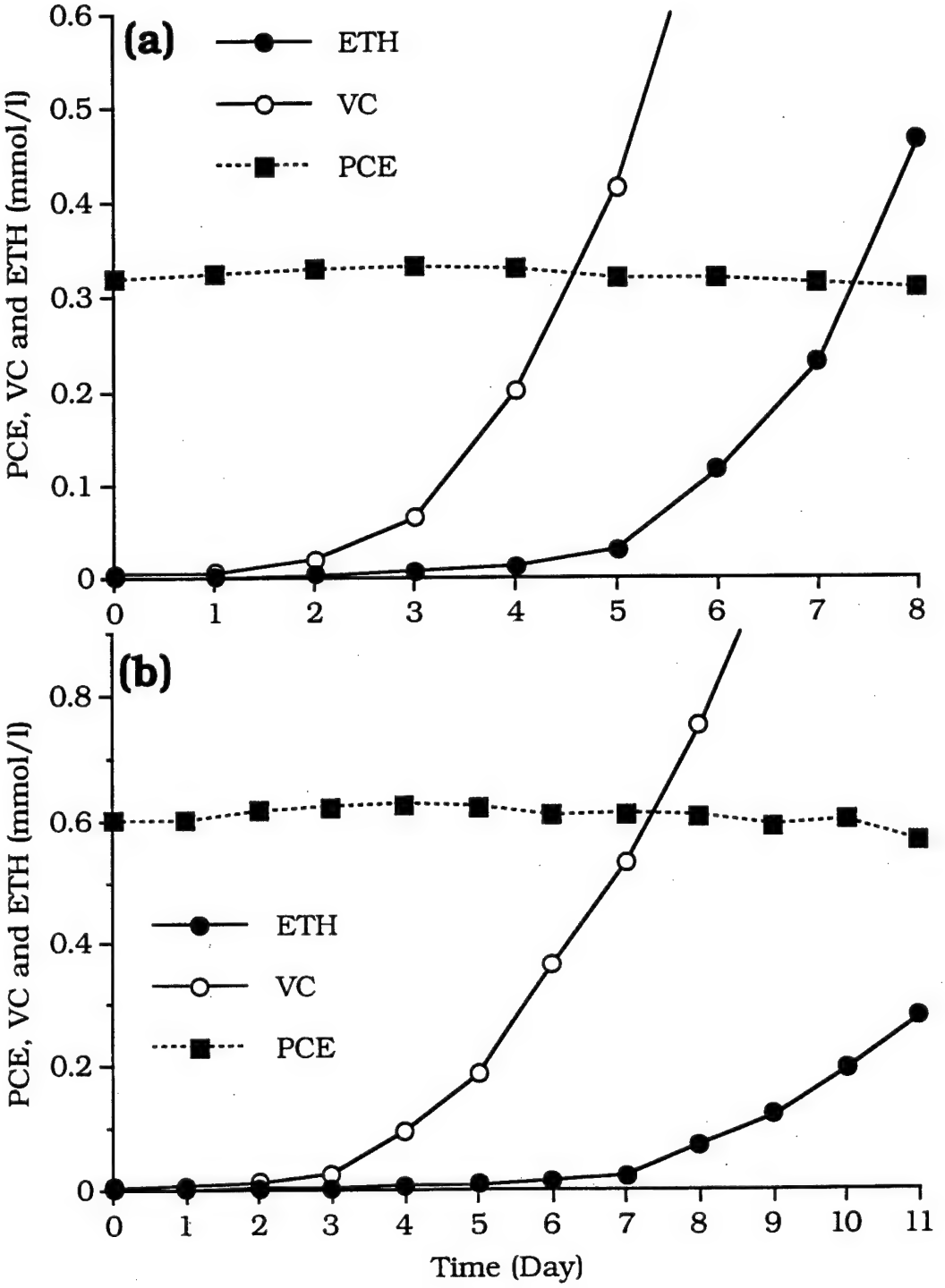


FIGURE 7.6 (a) Close up of Fig. 7.5 (a) and **(b)** close up of Fig. 7.5 (b) which shows ETH and VC production from PCE during the first 11 days of the experiment. TCE and DCE isomers have been omitted for clarity. The amounts of PCE, VC and ETH are shown as aqueous concentrations.

biomass to dechlorinate VC more rapidly as the density of the culture increases. This pattern of utilization of VC seems to be also typical of a cometabolic process, in which no energy is obtained from the compound, but the molecule in question is dechlorinated when a suitable growth substrate is utilized. Similar results were obtained previously by DiStefano *et al.* (8) in studies of the methanol/PCE culture while still in the methanogenic stage. The fact that the rates of ETH production diminished with time in experiments performed when PCE was not present {Fig. 7.1 (b) and 7.6 (a)}, reinforce the view that VC is cometabolically dechlorinated to ETH. Also, as found for *trans*-DCE in Chapter Five, the initial rates for ETH production obtained from a culture that had grown on PCE were much higher (0.043 mmol l^{-1} day^{-1}) than the ones calculated when VC was utilized as sole e^- acceptor (0.0015 mmol l^{-1} day^{-1}).

Fig. 7.1 shows that, in the culture transferred with VC only, the amount of ETH produced is 0.024 mmol/l. When examining at the culture that consumed a PCE dose right after transfer, the amount of VC present increased to 0.73 mmol/l, about two (1.9 to be exact) times higher than in VC-alone samples. From first-order kinetics, the rate of ETH formation should be 2-fold higher, so that 0.05 mmol/l ETH might be expected to accumulate over a similar time period in Fig. 7.1 (b). Instead, 0.18 mmol/l ETH accumulated. Simple dechlorination kinetics do not explain the VC degradation patterns. To understand it, we have to look at growth kinetics. *"D. ethenogenes"* strain 195 could be growing twice as fast at the higher VC concentration (due to growth on PCE), so that after several doublings

there would be a big difference. By comparison with results obtained in Fig. 5.8, the culture of Fig. 7.1 (b) could have doubled 3 to 4 times. Increases in biomass are therefore important in the dechlorination of VC by ""*D. ethenogenes*"" strain 195, a compound that usually accumulates in anaerobic aquifer systems due to its generally slow degradation (4, 5). The availability of required or appropriate nutrients is then of great importance to the degradation of this and other recalcitrant compounds.

As discussed in Chapter Five of this dissertation, VC and *trans*-DCE are both dechlorinated very similarly by *"D. ethenogenes"* strain 195 but, contrary to *trans*-DCE, VC plays a fundamental role in the dechlorination of PCE to ETH, where it is the main intermediate accumulated. The last step of dechlorination, VC to ETH, is the rate-limiting step to the complete dechlorination of PCE, in accordance with previous results (20, 21). This is in contrast to what it is found in all other pure cultures, in which TCE and *cis*-DCE (the latter as the final product) are accumulated (13, 15, 18, 22, 23); in mixed cultures which dechlorinate PCE to ethane, in which *cis*-DCE is accumulated to greater extent than VC (7); and in *in situ* studies, were DCEs are again accumulated to very significant extent (4, 19).

Because of the decrease of rates of VC dechlorination with time, the majority of ETH is produced during the first few days when all other chloroethenes (except *trans*-DCE) are absent. As seen in Fig 7.3 (c), there is not much difference in ETH production between 2 days and 4 days without PCE. The best conversion of VC to ETH occurs in a dense culture that has been growing on a suitable chloroethene as

sole e^- acceptor, in the absence of this or any other chloroethene utilized as growth substrate (Fig. 3.9).

The use of hexadecane (HDC) as a solvent for delivering PCE to the culture was investigated. This technique was devised by Holliger *et al.* (16) and it has been utilized with HDC and silicone as solvents in other studies (1, 10). HDC is a good solvent because it is essentially non metabolizable under anaerobic conditions, and it is considered non-toxic to many microorganisms. This solvent forms a separate phase, and, because of the high solubility of PCE in HDC, allows the addition of high amounts of PCE while keeping its aqueous concentration low. This ability to add a large amount of PCE at once means that frequent PCE additions are not necessary, and that the culture does not go long periods in the absence of PCE. It is important to add enough buffering capacity so that conversion of large amounts of PCE added does not acidify the culture via HCl production.

Several experiments described previously in this dissertation showed the ability of the culture to produce ETH in the presence of small quantities of PCE in the medium. Fig. 7.5 clearly shows that maintaining a relatively low (about 0.3 mmol/l), constant amount of PCE in the sample indeed allows for continual ETH production as the culture grows. PCE dechlorination and ETH production may therefore coexist at "low" PCE concentrations. Higher amounts of PCE (0.6 mmol/l), although they don't completely inhibit ETH production, limit this ability considerably. The diminishing production of VC and ETH after days 24 and 17, respectively, indicates the presence of a

limitation in the culture's ability to dechlorinate. Byproduct toxicity (salinization by production of NaCl and/or acidification by production of HCl) or nutrient limitation/s are good candidates for this loss of dechlorinating activity. It is interesting to note that cultures amended with a lower constant PCE concentration (0.3 mmol/l), dechlorinated more of this substrate than cultures with a higher constant level of PCE (0.6 mmol/l).

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APPENDIX A

VIRUS-LIKE PARTICLES ASSOCIATED WITH "*Dehalococcoides ethenogenes*" strain 195

"Abbott: Now, on the St. Louis team we have Who's on first, What's on second, I Don't Know is on third.
Costello: That's what I want to find out."
Bud Abbott 1895-1974 & Lou Costello (Louis Francis Cristillo) 1906-1959, in the 1945 film Naughty Nineties.

As was described in Chapter Three of this dissertation, *"D. ethenogenes"* strain 195 was able to grow and be isolated in the presence of ampicillin thanks to the required nutrients present in the cell extract from the mixed butyrate/PCE culture. After isolation, *"D. ethenogenes"* strain 195 grew and dechlorinated PCE to VC and ETH at high rates for the first two transfers (data not shown). During the second transfer, the culture had been fed more PCE doses than ever before, to determine the dechlorination limits of this organism under the present conditions. The doses of PCE consumed consecutively were: 0.4, 0.6, 0.8, 1.0, 1.2, 1.4, 1.6, 1.8 mmol/l PCE (total of 8.8 mmol/l PCE in nominal concentration, in 12 days). *"D. ethenogenes"* strain 195 showed no limitation of dechlorination. All PCE doses, including the last one (which is just below the limit of solubility in water for PCE), were transformed almost stoichiometrically to VC and ETH, without signs of substrate or product inhibition.

This rapidly dechlorinating culture was then transferred a third

time into fresh medium ("Transfer #3"). Contrary to the first two transfers and against all expectations, the newly transferred culture was unusually slow in dechlorinating PCE (Fig. A.1). It took 10 days to dechlorinate the first dose of PCE (0.3 mmol/l), about twice the time expected, and there was substantial accumulation of intermediates (TCE and DCEs), a sign that the culture was limited in some way. There was no apparent explanation for the sudden slow down of the culture's ability to dechlorinate. All the conditions in which the culture had been transferred were identical to the ones in the past two transfers, and *"D. ethenogenes"* strain 195 should have grown and dechlorinated in no different manner. Several initial hypothesis were presented:

1). An error in the preparation of the media or the buffering system (HCO_3^-/CO_2) utilized could have caused the pH to be off the neutral zone. The pH in the culture was found to be at 6.9.

2). The cell extract obtained from the mixed butyrate/PCE culture (the key ingredient in growing *"D. ethenogenes"* strain 195 axenically) has a limited time span of activity. After 4 to 7 weeks at 4°C, the extract gradually loses its ability to sustain dechlorination in the axenic culture, probably due to degradation or inactivation of the active ingredient(s) that it contains. Because the extract utilized for the third transfer had been at 4°C for about 3.5 weeks, it seemed plausible that the slow down in the culture's ability to dechlorinate PCE could be due to an "aging" cell extract.

To determine the potency of the cell extract amendment, a different culture (a culture grown for the first time in ampicillin) was

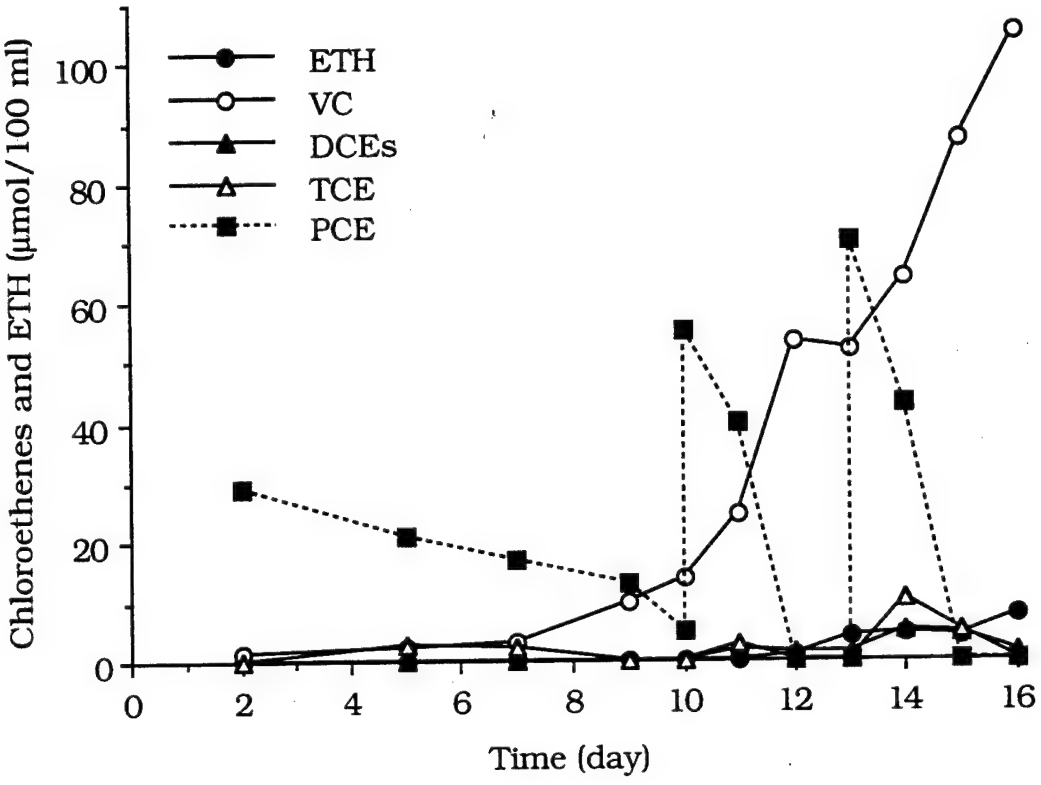


FIGURE A.1 Products of dechlorination from PCE in a third generation pure culture of *"D. ethenogenes"* strain 195 grown in the presence of ampicillin and with the ABSS and the extract from the butyrate/PCE culture as nutrients.

transferred with the same cell extract. Although dechlorination gradually stopped after 3 PCE doses (sign of limitation), during the first 10 days the rates of dechlorination were as fast as usual and the culture showed no limitation of dechlorinating activity (data not shown). Then, however some activity of the extract may have been lost during storage, this fact alone could not account for the slow initial rates of PCE dechlorination by Transfer #3.

Fig. A.2 shows seven of the photomicrographs obtained from Transfer #3 when it was observed under transmission electron microscopy by negatively staining the cells of *"D. ethenogenes"* strain 195. Only one bacterial type corresponding to *"D. ethenogenes"* strain 195 was found in the samples. Surprisingly, the vast majority of cells seemed to be heavily and actively infected by a particular kind of virus-like particle. No isolation or in depth studies of these particles were performed, but it is clear that morphologically they closely resembled the MVL1-type viruses (Mycoplasmatales Virus *laidlawii* 1) of Acholeplasmas, in the Class Mollicutes (also known with the generic name of Mycoplasmas) (1, 2, 3).

Mollicutes are the smallest free-living, self-replicating bacteria known (6). These organisms (mainly from the genus *Acholeplasma*, from which the MVL1 virus was first isolated) share an important number of characteristics in common with *"D. ethenogenes"* strain 195 (5, 6), even though their nutritional requirements and 16S rRNA derived phylogenies are quite different. Acholeplasmas share with *"D. ethenogenes"* strain 195 their small size (0.3 to 0.8 μm); the lack of a peptidoglycan cell wall (and, thus, their pleomorphicity and resistance

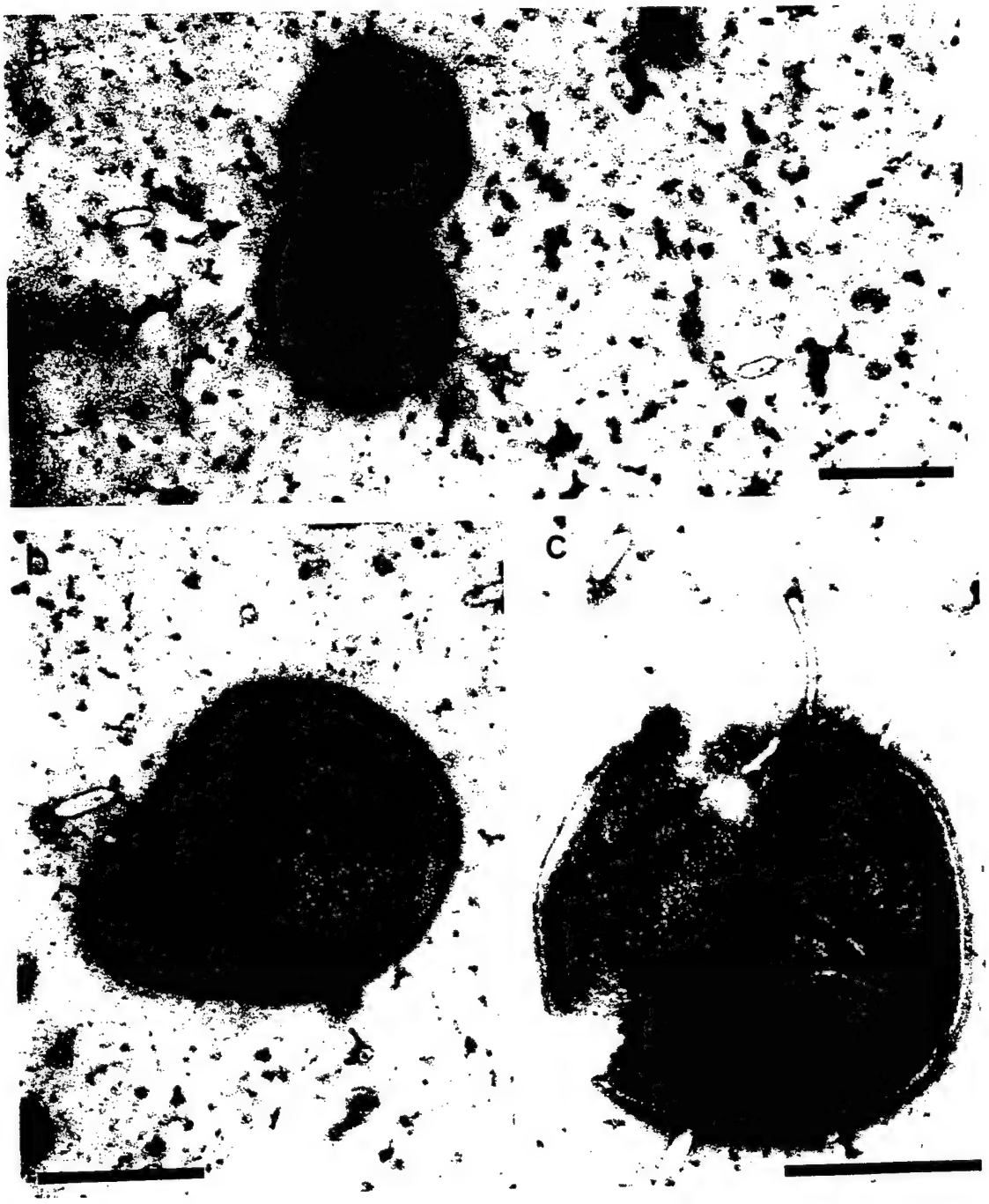
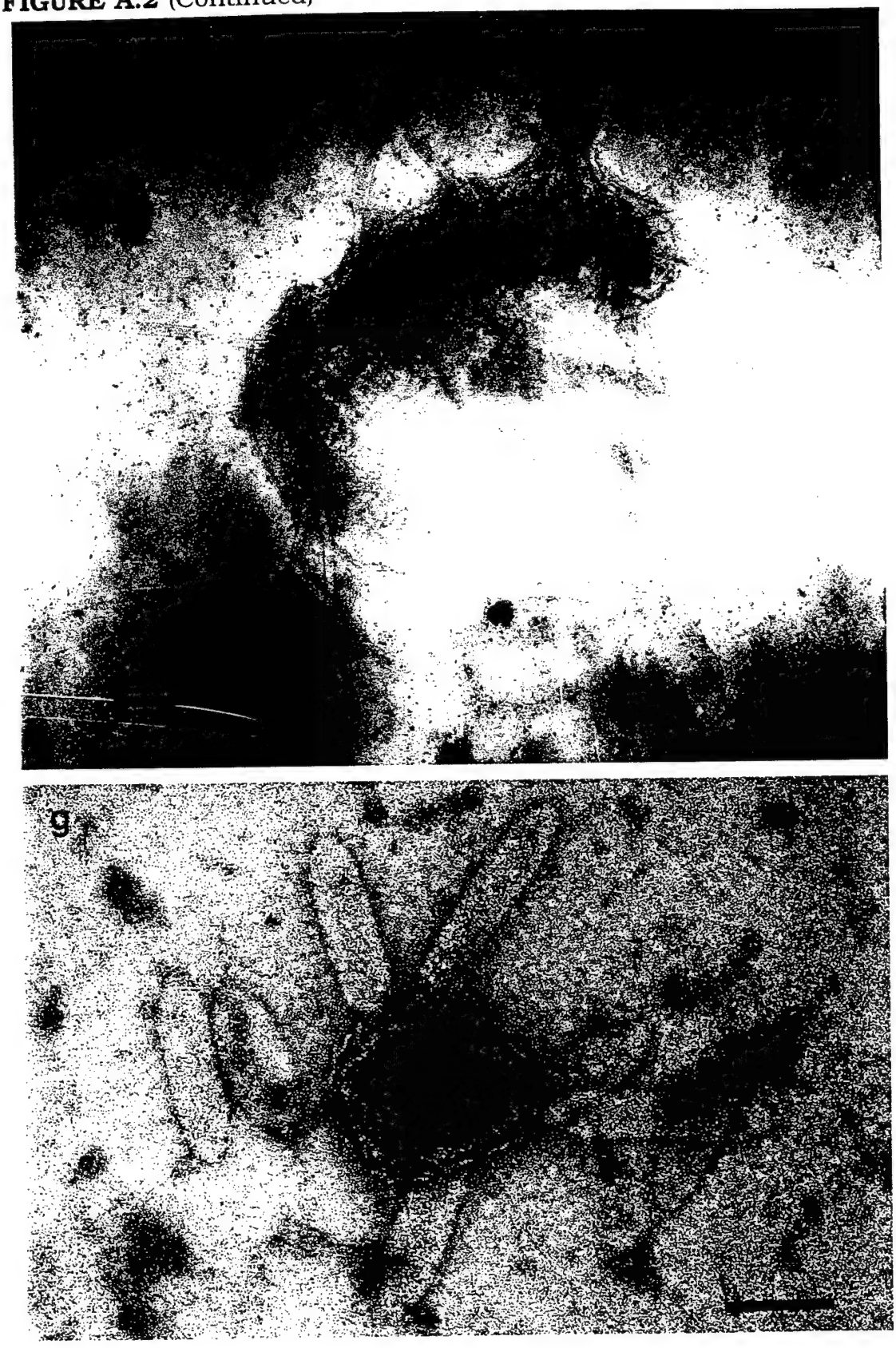


FIGURE A.2 Negatively stained (2% UA) preparations of *"D. ethenogenes"* strain 195. **(a)** and **(b)** show, respectively, two and one potentially non-infected cells surrounded by bullet-shaped viral particles. **(c)** Shows an infected, lysed cell. **(d)** Shows a non-lysed, infected cell. **(e)** Shows three very infected cells, one of which has lysed. **(f)** Shows a very infected cell with numerous viral structures extruding from the cell. **(g)** Shows a cluster of bullet-shaped viral particles attached to a membrane. Marker bars represent: 0.5 μm **(a)-(e)**; 0.2 μm **(f)**; and 0.1 μm **(g)**.

FIGURE A.2 (Continued)

FIGURE A.2 (Continued)



to peptidoglycan targeted antibiotics); non motility; multiplication by binary fission; fastidious growth; and, as *"D. ethenogenes"* strain 195, they were first isolated from sewage.

MVL1 viruses are naked rod- or bullet-shaped particles that are assembled at and protrude from the surface of the infected cell (1). The mechanisms of viral DNA penetration into the host are unknown. The infection is non-lytic, which leads to persistently infected cells, and virus maturation and release are coupled and involve interactions with the cell membrane which do not seem to affect viability, although infected cells grow more slowly than do uninfected control cells (4). Evidence indicates that some whole viral genomes are integrated into the host chromosome (6). Normal infectious particles measure 13-16 by 80-90 nm, and they have been observed to be twice as long occasionally. This virus contains single-stranded, covalently closed circular DNA with a molecular weight of $1.5\text{-}2\text{x}10^6$ (2, 3).

The photomicrographs taken from the slow-growing, Transfer #3 of *"D. ethenogenes"* strain 195 of Fig. A.2 showed the typical pleomorphic coccoid shape with size ranges from 0.6 to 1 μm in diameter. No contaminating bacteria were present in the samples. Fig. A.2 (a) and (b) show, respectively, two and one apparently non-infected cells surrounded by bullet-shaped viral-like particles. Non-infected cells were very rare and constituted a very small proportion of the total number of cells in the samples. The sizes of the viral-like particles ranged from 35 to 60 nm in width and 100 to 200 nm in length. This is also seen in better detail in Fig. A.2 (g), which shows, at higher magnification, a cluster of bullet-shaped viral-like particles

attached to a fragment of membrane {this is also seen at the right and left lower part of Fig. A.2 (d)}. Fig. A.2 (c), (e) and (f) show cells that had been heavily infected. Two of the cells shown had lysed and still showed viral particles attached to their membranes, as did all other infected cells. Most of the cells encountered in the study, although heavily infected, were not lysed. This fact opens the possibility for these cells to be metabolically active, though with low levels of activity, which would account for the low levels of dechlorination measured in Fig. A.1. Two types of viral-like particles could be seen in the micrographs from Fig. A.2: bullet-shaped particles of regular structure that were found free in the medium or attached to the cell envelope; and elongated irregular rod-like particles that were only found protruding from cell envelopes {see Fig. A.2 (f)}.

To determine that this overall heavily infected culture was a finding unique to the slow-growing, Transfer #3 of *"D. ethenogenes"* strain 195, several other cultures that dechlorinated at normal fast rates were negatively stained and observed under electron microscopy (data not shown). A sample from a mixed culture that grew on H_2/PCE and had been at 4°C for 14 months showed non-infected cells in all observations. A very low number of bullet-shaped viral particles were found, and none of them attached to any cell. Another sample from a mixed culture that also grew on H_2/PCE and had been at 4°C for over 2 years also showed all cells to be free of viruses. In this case, no bullet-shaped particles were found. No elongated rod-shaped viral particles were found in either culture. Both cultures contained rod-shaped cells (the other main morphology in the mixed culture; see

Chapter Two) as well as *"D. ethenogenes"* strain 195 cells. In both samples as well, several other types of viruses were found, as it can be seen in Fig. A.3.

A sample of the culture that had been utilized as inoculum for the heavily infected culture (Transfer #3) was also negatively stained and observed under the electron microscope (data not shown). Only cells of *"D. ethenogenes"* strain 195 were found, at a very high cell density (when compared to any of the other samples). There were no lysed cells nor heavily infected cells and the culture appeared perfectly "healthy", except for the fact that almost each and every cell had a bullet-shaped particle attached to the cell envelope. Only a few of the cells showed more than one virus attached. There were no elongated rod-shaped morphologies on cells, seen so profusely in the actively infected culture.

The actively infected, slow dechlorinating culture (Transfer #3) was transferred into fresh medium only after the third dose of PCE had been consumed (at Day 16). Fig. A.4 shows the pattern of dechlorination exhibited by this overall fourth transfer of the pure culture. Surprisingly, the pattern and rates of PCE dechlorination were as fast as they had ever been up to that point. PCE was dechlorinated to VC and ETH faster with time and with very little accumulation of intermediates. If a viral infection had been the cause of the slow down in the culture's ability to dechlorinate PCE in the previous transfer, at this point the phase of infection had to have past. If true, we should not find actively infected cells. Negative stains

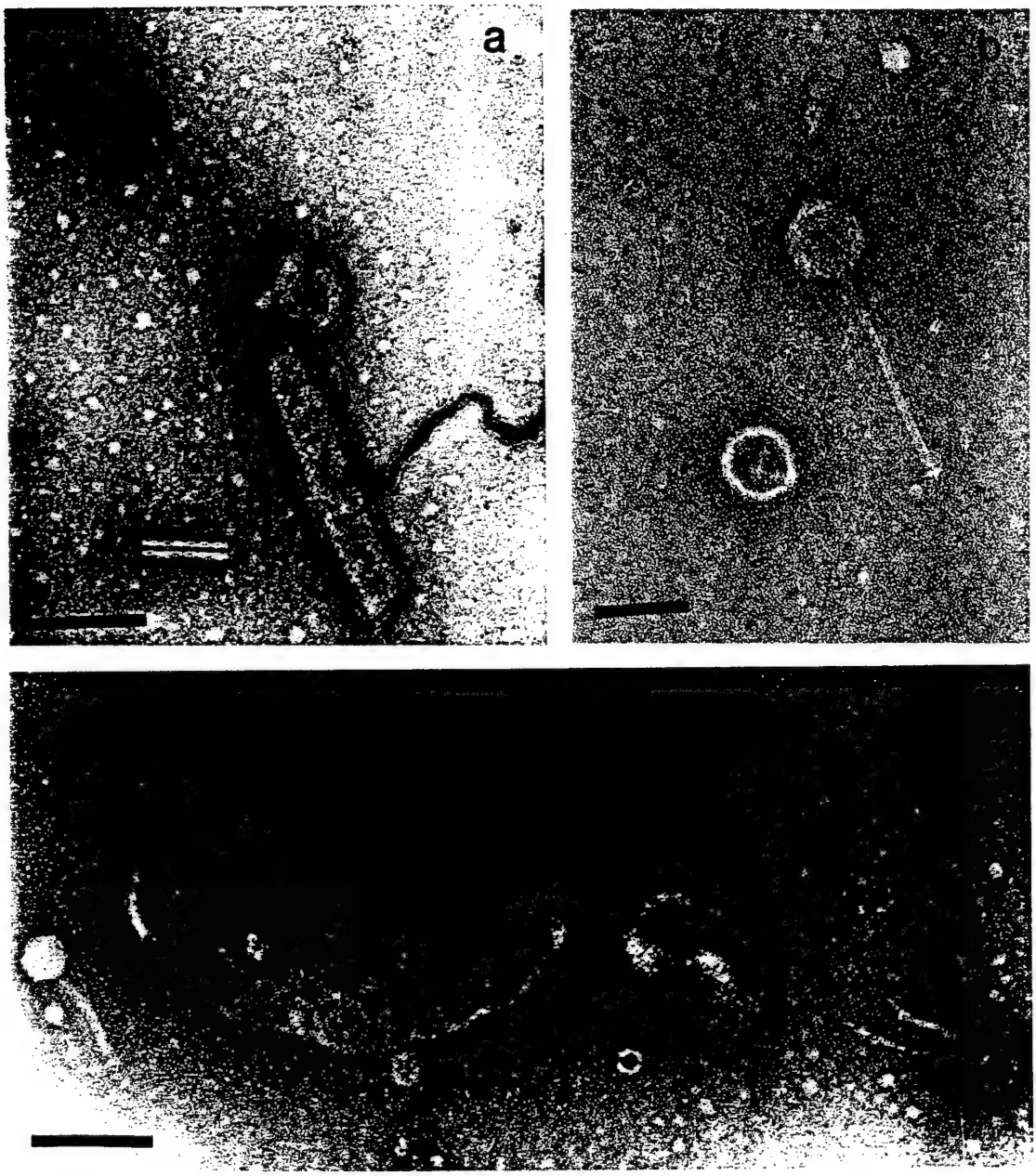


FIGURE A.3 Negatively stained (2% UA) viral particles obtained from preparations from purified mixed H_2/PCE cultures. Bars are: **(a)** 0.2 μm; **(b)** 0.1 μm; and **(c)** 0.2 μm.

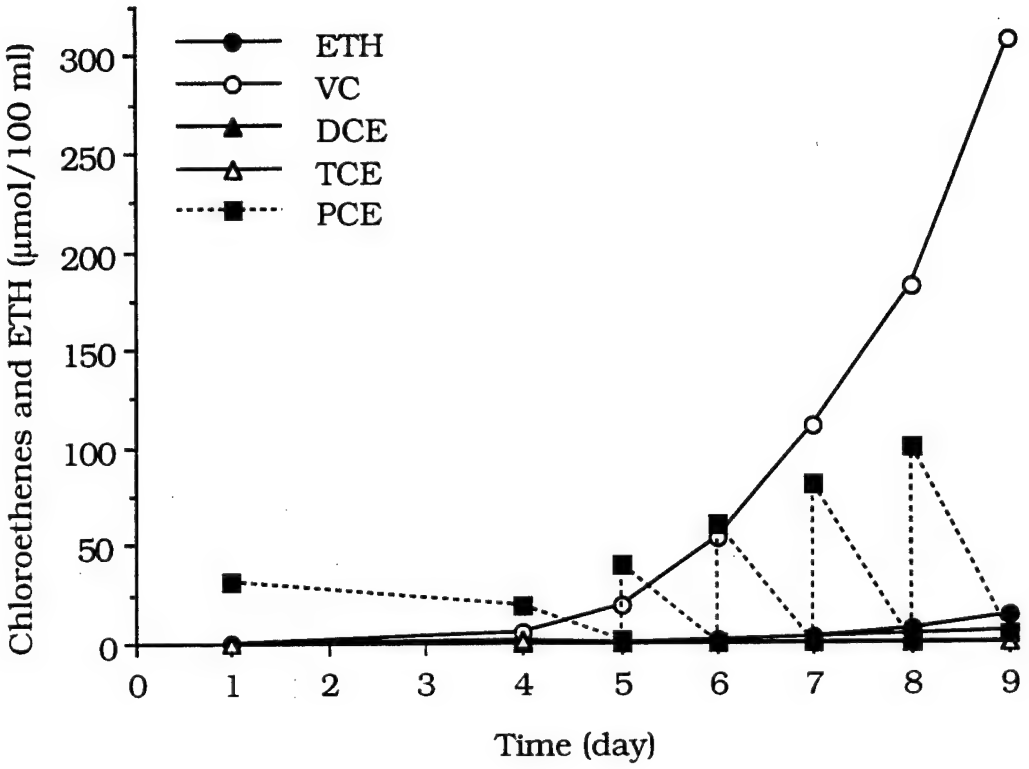


FIGURE A.4 PCE dechlorination and product formation by *"D. ethenogenes"* strain 195 transferred from the heavily infected, slow-dechlorinating culture (transfer #3).

of this culture showed coccoid cells only, reaffirming the purity of the culture, which appeared very healthy with only a few bullet-shaped viruses present, none of them attached to any cell (data not shown), confirming the prediction. This culture was transferred again with identical good growth and PCE dechlorination patterns. No further phase of aggressive viral infection like the one described above has been found again and cultures have grown and dechlorinated PCE at fast rates since.

These viral particles could have been present in the culture since its initial enrichment some years before I started working in this project. Nevertheless, they could also have been brought in by a complex amendment like the sludge supernatant (SS), which was added to the culture as a required nutrient. When a sample of SS was negatively stained and observed under electron microscopy, no bullet-shaped viruses were found (although polyhedral and round viruses were observed; data not shown)). The cell extract from the mixed butyrate/PCE culture, also added as a required amendment, was then examined. A sample from the first extract produced and another sample from the newer batch of extract yielded no bullet-shaped virus particles under electron microscopy (data not shown).

Even though there may be many explanations for the potentially heavily infected culture showing slow dechlorination rates, and there is no assurance that the viral infection had a direct relationship with the slowdown of the culture, an explanation relating the cause of the slowdown to viral infection appears plausible. Although the origin of these viral particles is unknown, it is

clear that they were present in the culture at least 14 months before the isolation of *"D. ethenogenes"* strain 195, which occurred in January 1995. In accordance with the results presented in this appendix (combined with what is known about MVL1-type viruses) an overall possible explanation for the spectacular transitory infective phenomenon could be as follows:

The virus-like particles can be found in two different morphologies: bullet- and rod-shaped. Bullet-shaped particles are found free in the medium and, in this form, the virus attaches itself to the cell envelope and starts the process of invasion by inserting its DNA through a peptidoglycan-free envelope. The fact that free bullet-shaped particles are seldom seen in fast dechlorinating cultures does not mean that the viruses are not there. When the bullet-shaped particles infect a cell, their DNA could become integrated into the genome of the cell and remain there until a particular stress (cell density, nutritional deficiency, product toxicity, etc.) or a combination of stresses would trigger an active cycle of viral infection. This is what could have happened at the time the Transfer #3 tubes were inoculated. A different morphological state of the same virus, the elongated rod-shaped structures, are always found associated to cell envelopes. Contrary to the bullet-shaped form, which always rests on the cell envelope, these rods protrude from the inside of the cell (see Fig. A.2). These rod-shaped structures seem to be the extruding form of the bullet-shaped virus. These elongated rod-shaped particles seem to be surrounded by the cell membrane and in them the new viral particle may be assembled. It is obvious from Fig. A.2 (e) that some of

these rod filaments contain a bullet-shaped virus at their outermost tip, which is still connected to the cell by a thinner bridge, probably formed by the cell envelope itself. At a particular point in time, the bullet-shaped virus detaches itself from the cell, becoming free moving in the medium. If the cycle is non-lytic, this would account for the slow growth and dechlorination observed in the infected culture (Transfer #3). The faster growth rates of non-infected cells with respect to infected ones could, by itself, diminish a generalized infective process and turn the balance towards an overall non-infected, fast growing and dechlorinating culture (this could be a reason why a transfer of Transfer #3 behaved and looked normally).

If this hypothesis is true, some kind of stress triggered the infectious wave in the culture that had just then been utilized as inoculum for the transfer that proved to be heavily infected. The possibility that the triggering of this infectivity could be cell-concentration dependent is not negligible. The culture used as inoculum for the heavily infected transfer was unusually dense. A 10% v/v extract from the butyrate/PCE culture was used for the first time (usually a 5% v/v was used) and the response of the culture to it was extraordinary. *"D. ethenogenes"* strain 195 consumed more PCE doses faster than ever before, with the consequence that the cell density was the highest we had ever gotten (considering that this was a pure culture).

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APPENDIX B

NUTRIENT STUDIES: SLUDGE SUPERNATANT (SS)

"Shome mishtake, shurely?"
Catch-phrase in Private Eye magazine, 1980s.

Preliminary studies on the SS have been described in Chapter Two of this dissertation, before the isolation of *"D. ethenogenes"* strain 195 had been accomplished. In the pure culture, experiments to try to substitute the SS with a better defined amendment were performed, with mixed results. Cultures growing with acetate, SS and cell extract from the mixed butyrate/PCE culture were transferred into fresh medium in the presence or absence of SS, in the presence or absence of the cell extract, and into medium in which the SS had been substituted by the following mixture of volatile fatty acids (VFA; in parenthesis are their final concentrations in mM): Propionate (8); butyrate (3.2); valerate (0.92); acetate (29.7); isovalerate (0.91); isobutyrate (1.09); and 2-methyl-butyrate (0.82). As seen in Fig. B.1, the presence or absence of these particular VFA solution did not influence the dechlorination patterns of the culture. In a different experiment, the same mixture of VFA did not sustain dechlorination nor growth of the culture in the absence of SS after 32 days (data not shown). As Fig. B.1 shows, cultures containing SS and lacking cell extract did not sustain dechorination. On the contrary, though, cul-

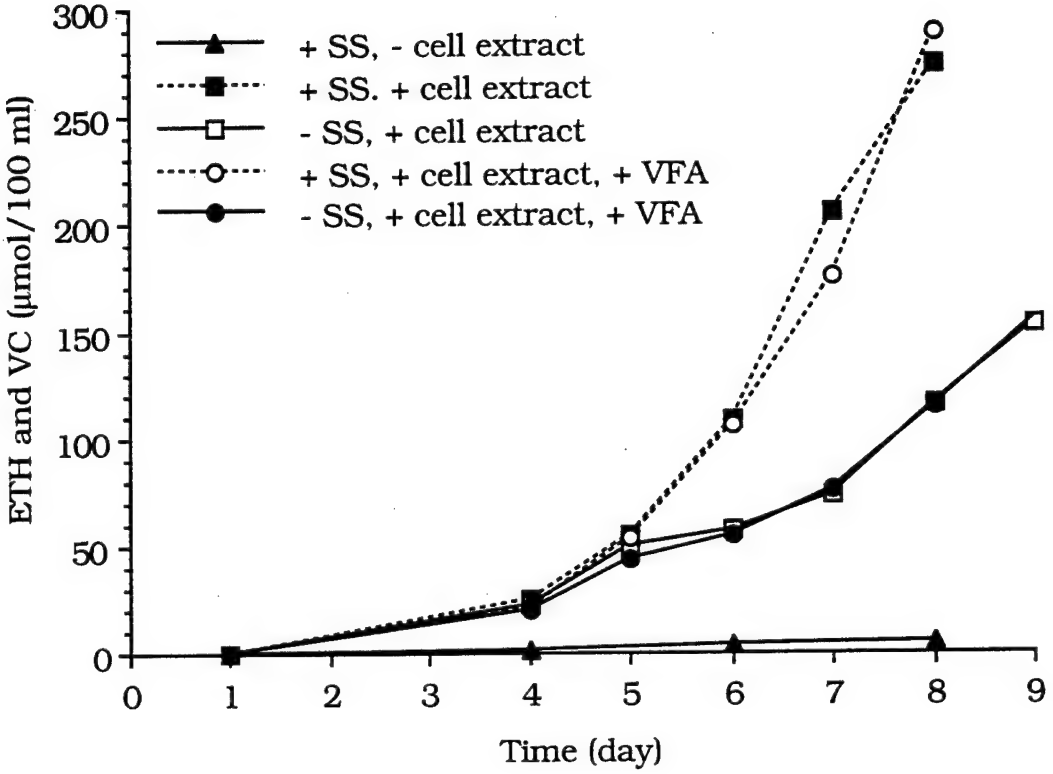

FIGURE B.1 Cultures that had been growing with acetate, SS and cell extract from the mixed butyrate/PCE culture were transferred into fresh medium in the presence or absence of SS, in the presence or absence of the cell extract, and into medium in which the SS had been substituted by the next mixture of volatile fatty acids (VFA; in parenthesis are the final concentrations in mM): Propionate (8); butyrate (3.2); valerate (0..92); acetate (29.7); isovalerate (0.91); Isobutyrate (1.09); and 2-methyl-butyrate (0.82). The graph shows VC and ETH formation from PCE by these transfers.

tures containing cell extract and no SS dechlorinated PCE to VC and ETH with no accumulation of intermediates, although the rates were slower than in controls containing both amendments. This culture lacking SS (containing cell extract) was transferred four more times under the same conditions (data not shown). Although dechlorination was sustained in all transfers, each time the culture was transferred, the accumulation of intermediates (TCE and DCEs) increased. In the last transfer, almost no ETH was produced and both TCE and DCE isomers accumulated to elevated amounts when compared with the control cultures containing SS.

On one hand, from results obtained in Chapter Three of this dissertation, it is clear that the cell extract contains one or more nutrients, not present in the SS, that are required by the culture in order to grow and dechlorinate PCE axenically. On the other hand, from the results of Fig. B.1, the cell extract is able to partially substitute for the SS, which means that at least one of the required nutrients present in the SS is also present in the cell extract.

To determine the possibility that the required nutrient(s) in the SS that was not present in the cell extract was an amino acid, a sterile, vitamin free Bacto Casamino acids (CA; Difco, Detroit, MI) solution was prepared anaerobically to be added in substitution of the SS. The CA solution contains all the amino acids except for asparagine, glutamine, cysteine and tryptophan. Fig. B.2 shows the results of an experiment in which *"D. ethenogenes"* strain 195 was transferred into eight sets of tubes under different conditions. All tubes contained cell extract, and all were tested with and without the

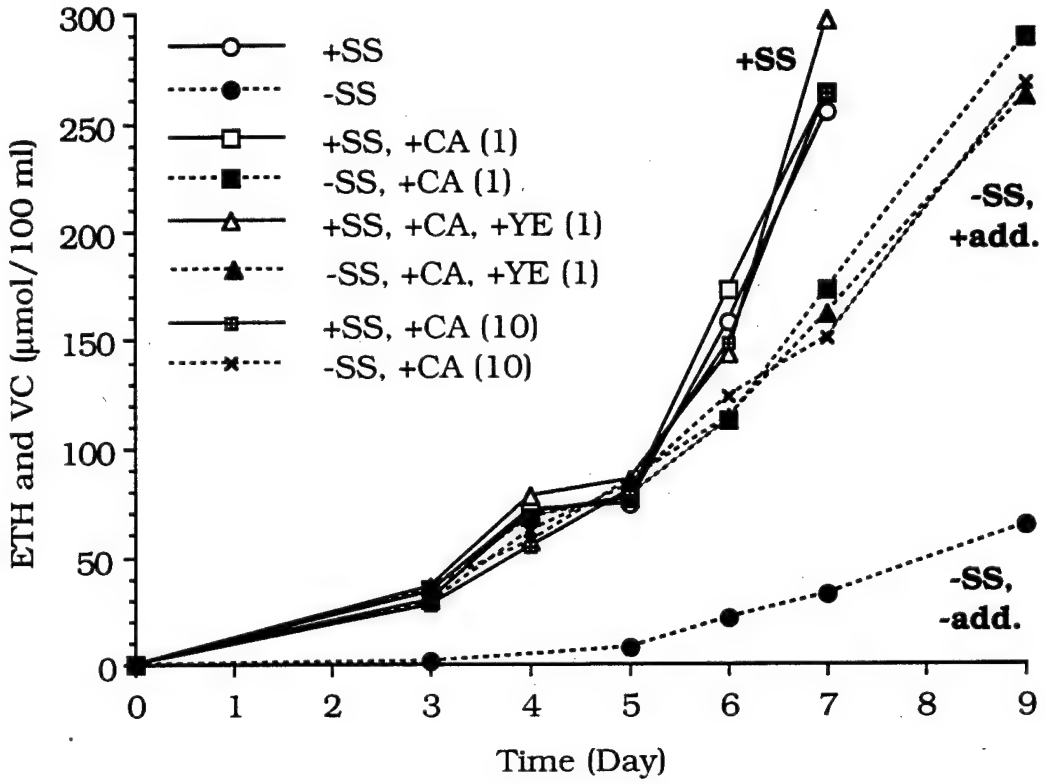

FIGURE B.2 Product formation (ETH and VC) from PCE by *"D. ethenogenes"* strain 195 transferred into eight sets of media under different conditions: All tubes contained cell extract from the mixed butyrate/PCE culture, and all were tested with and without the Casamino acids solution (plus or minus yeast extract), in the absence or presence of SS. Numbers in parenthesis represent amounts of amendments, in mg/ml.

CA solution (plus or minus yeast extract), in the absence or presence of SS. Three main responses can be observed in Fig. B.2. Cultures without SS dechlorinated PCE at low rates, as seen also in Fig. B.1. Cultures with and without SS dechlorinated PCE with similar rates during the first 3 days in the presence of casaminoacids (independently of the amount added). After that, cultures lacking SS showed lower rates of PCE dechlorination than cultures where SS was present. These cultures were all observed using phase-contrast microscopy and no contamination was found. At Day 4, cultures grown with SS had no PCE left, and a new dose was not administered until Day 5, hence the delay in product formation in these cultures from Day 4 to Day 5. This fact accounts for a greater difference in VC and ETH formation between cultures having and lacking SS than that showed in Fig. B.2. The results show that one or more amino acids are required by the culture. Nevertheless, because the rates of PCE dechlorination are still better in the presence of SS than with the CA solution, a different nutrient present only in SS must account for this difference. This nutrient, specific to the SS, is not a common nutrient that would be found, for example, in yeast extract, because in the presence of Casamino acids, yeast extract had no effect in the ability of the culture to dechlorinate.

One of the cultures grown with no SS and with the CA solution was transferred under the same and opposite (with SS and no CA) conditions. The results (data not shown) were very similar to the ones obtained in Fig. B.2. The cultures with SS instead of the CA solution had higher rates of dechlorination than did the ones having the CA

solution and no SS. The latter cultures dechlorinated PCE to VC and ETH slower but normally, without the accumulation of high quantities of intermediates (TCE and DCEs). The CA solution contained the next ingredients (Difco Laboratories, Detroit, MI):

L-Amino acids (%)	
Alanine	3.3
Arginine	2.2
Aspartic acid	4.8
Glutamic acid	15
Glycine	1.3
Cystine	0.16
Histidine	1.7
Isoleucine	3.3
Leucine	5.5
Lysine	5.7
Methionine	1.3
Phenylalanine	2.1
Proline	6.2
Serine	2.2
Threonine	2.4
Tryptophan	<0.01
Tyrosine	0.47
Valine	4.3

Inorganics (%)	
Chloride	7.4
Magnesium	0.002
Phosphate	3.33
Potassium	0.4
Sodium	8.7
Sulfate	0.045
Sulfur	0.042
Calcium, cobalt, copper, iron, lead, manganese, tin, zinc	<0.001

Nitrogen content (%)	
Total nitrogen	10.5
Amino Nitrogen	8.8

In conclusion, the CA solution could partially substitute for the SS (showing slower rates of dechlorination). Then, one or more of the amino acids present in this solution are necessary but not sufficient for the culture to dechlorinate PCE at fast rates axenically, or that a different ratio of concentrations of amino acids in the solution is needed. The other potential nutrient(s) present exclusively in the SS cannot be found in yeast extract nor in the VFA solution tested.

APPENDIX C

NUTRIENT STUDIES: SUBSTITUTION OF THE CELL EXTRACT FROM THE MIXED BUTYRATE/PCE CULTURE

"To ask the hard question is simple"
W.H. Auden 1907-73, Poems (1933) no. 27

Before the isolation of *"D. ethenogenes"* strain 195, the mixed H_2/PCE culture showed, apart from the dechlorinator, one other major morphotype in high numbers (a short rod) and a third less numerous morphology (a long rod) (see Fig. 2.9). Because *"D. ethenogenes"* strain 195 was originally isolated (see Chapter Three) with the cell extract from this mixed H_2/PCE culture, it was of interest to isolate the short rod to see if an extract from this organism would contain the nutrient(s) required to grow the dechlorinator axenically. The cell extract obtained from the mixed butyrate/PCE culture, which has been utilized to grow *"D. ethenogenes"* strain 195 since shortly after its isolation, has a limited time span of affectiveness (see Appendix A). Then, its substitution for a more defined, maybe more stable extract was of interest.

To isolate the short rod (named Strain DSR, for Dechlorinator Syntrophic Rod; work performed by Timothy Anguish), a mixed H_2/PCE culture was serially diluted. The lower dilution (10^{-6}) was

inoculated into agar roll tubes containing yeast extract (YE, 1 mg/ml) and SS and, once colonies were visible, a clean colony was picked and serially diluted again, down to 10^{-6}.

To obtain the best possible growth of Strain DSR, so that cell extracts would increase in their activity, an initial characterization of this organism was performed. Strain DSR is a Gram-positive short rod in stationary phase, but is longer and more motile during the growth phase. This organism was grown under different conditions and the O.D. was measured at 600 nm after 54 h (Table C.1).

Strain DSR did not grow on glucose, but grew, as expected, on YE. Increasing concentrations of YE favored more growth of this organism, and YE seemed to partially substitute for the SS requirement that this organism also had. Growth was stimulated by addition of Casamino acids (CA). The best growth was obtained when both YE and CA were present at high concentrations, as observed also under phase contrast microscopy.

The ability of YE to make the requirement for SS unnecessary was investigated further (data not shown). Strain DSR growing with 2 g/l YE and no SS was transferred into medium containing YE and CA, both at 5 g/l. After 43 h, cultures containing SS were as dense as cultures without it (O.D. at 600 nm of 0.545 and 0.552 respectively) and both showed no contamination when observed under phase contrast microscopy. These cultures were transferred several times without SS. Thus, strain DSR can grow with or without SS.

Now that a good nutrient supplement had been found for Strain DSR, this organism could grow to high density in a short time.

TABLE C.1 Effects of adding different nutrient amendments on the growth of Strain DSR, as measured by optical density (600 nm after 54 hours of incubation).

Amendment	*O. D. (600 nm)*
YE (2 g/l)	0.186
YE (2 g/l)-SS	0.113
YE (5 g/l)	0.360
CA (2 g/l)	0.086
Glucose (20 Mm)	0.008
YE (2 g/l)+CA (2 g/l)	0.345
YE (5 g/l)+CA (5 g/l)	0.653

Therefore, a cell extract from Strain DSR was tested for its ability to substitute for the extract from the butyrate/PCE culture. The extract from Strain DSR was prepared by the same procedure as the one used for the butyrate/PCE culture described in Chapter Three (by rupture with a French pressure cell and collection of the supernatant after centrifugation at 17,000 rpm (39,000 x *g*) for 30 min.). Fig. C.1 shows that an extract from Strain DSR grown on YE and CA allowed poor growth and dechlorination when compared to the cell extract from the butyrate/PCE culture, the standard required amendment. Also, the culture provided with the Strain DSR extract did not produce any ETH by Day 11. To investigate this phenomenon further, we examined both the particulate and the supernatant fractions of the extract from Strain DSR after centrifugation (Fig. C.2). Both fractions had better activity than no addition, and poorer activity than the butyrate-culture extract, but substantially more PCE dechlorination occurred in cultures amended with the particulate fraction of the culture — almost as good activity as the butyrate-culture extract. This suggests that the factor(s) in the rod culture are less soluble than they are in the butyrate culture, in which we have found little activity in the particulate fraction. There is some congruence between these findings and our previously reported finding that the factor(s) are retained by a 50,000 MW filter (see Chapter Three of this dissertation).

According to the protocol followed to make both extracts (described in Chapter Three), the last centrifugation was done at 17,000 rpm. To determine if a slower spin would maintain better activity in the supernatant fraction of the extract from Strain DSR, a

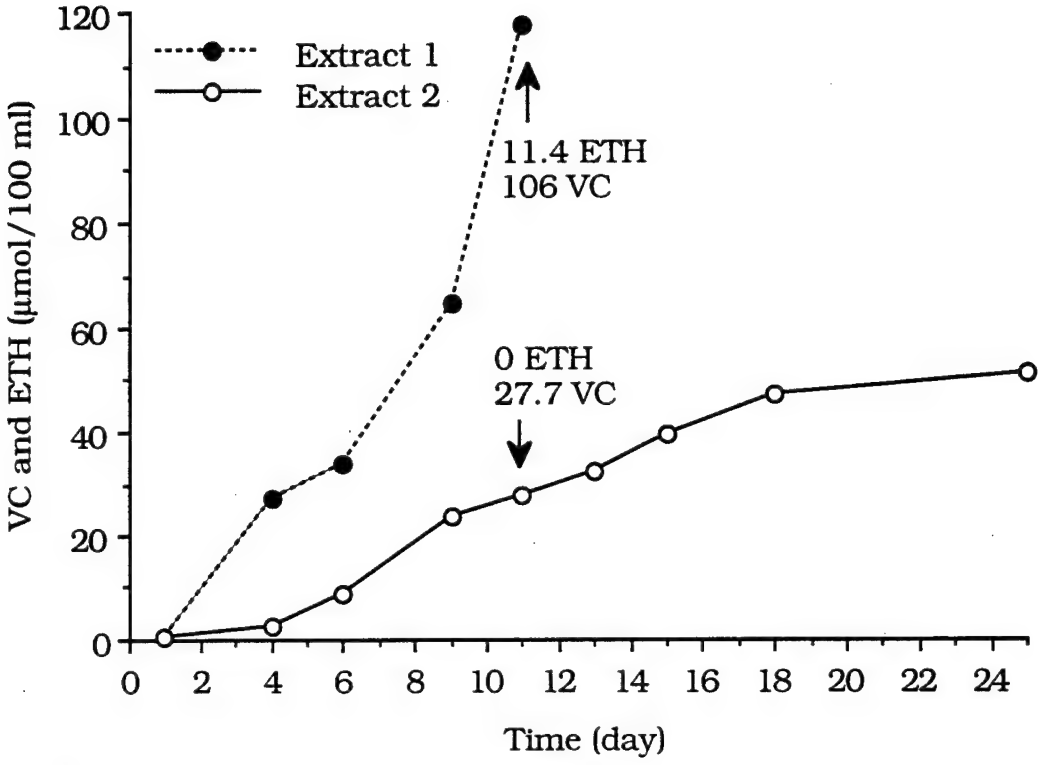

FIGURE C.1 VC and ETH production by *"D. ethenogenes"* strain 195 when grown axenically with different cell extracts. Extract 1 = from butyrate/PCE culture; extract 2 = from Strain DSR.

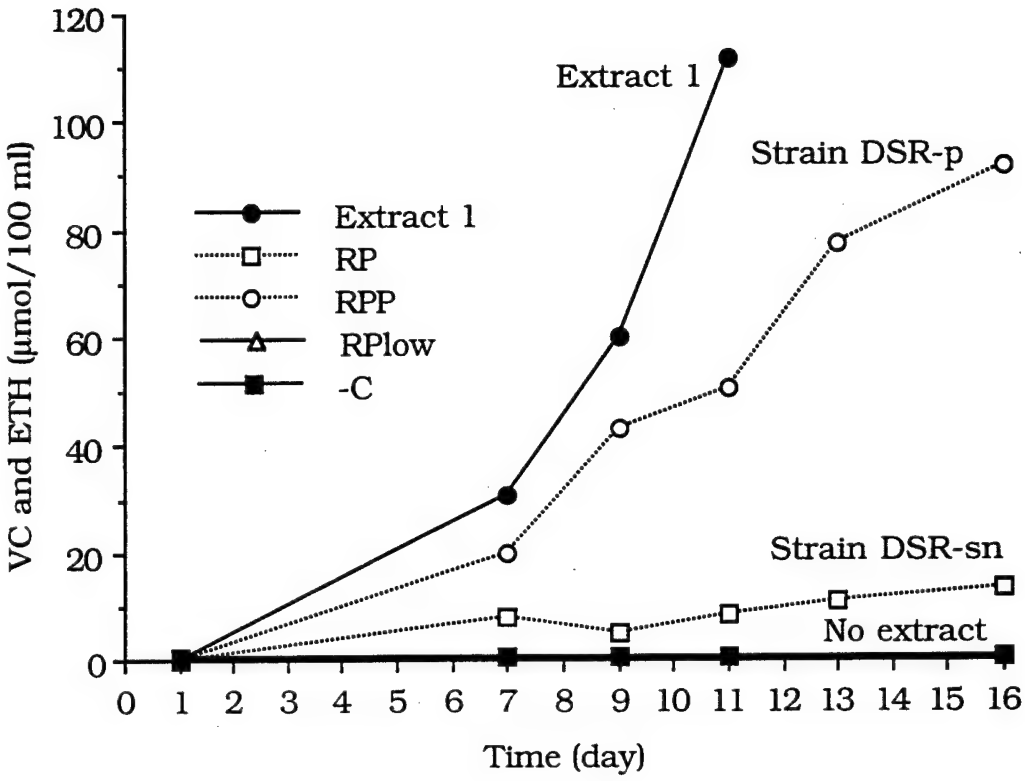

FIGURE C.2 VC and ETH production by *"D. ethenogenes"* strain 195 when grown axenically with different cell extracts. Extract 1 = from butyrate/PCE culture; Strain DSR-p = pellet fraction of an extract from Strain DSR; Strain DSR-sn = supernatant fraction of an extract from Strain DSR.

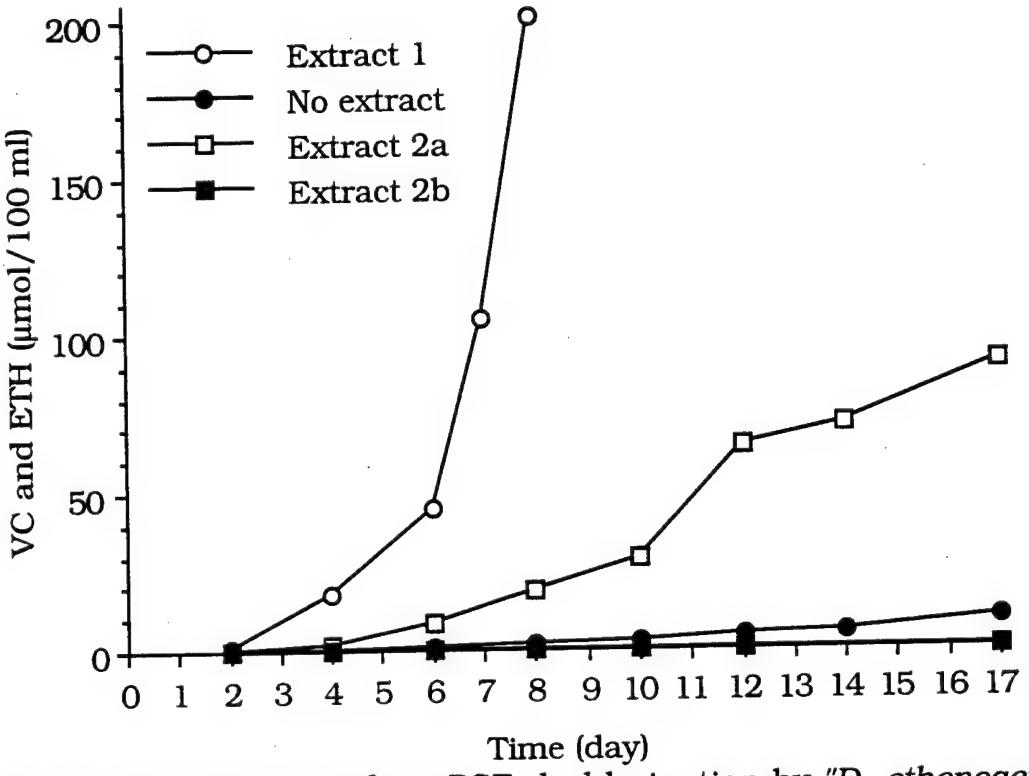

FIGURE C.3 Products from PCE dechlorination by *"D. ethenogenes"* strain 195 when grown axenically with different cell extracts. Extract 1 = from butyrate/PCE culture; extract 2a = pellet (particulate) fraction of an extract from Strain DSR; extract 2b = supernatant fraction of an extract from Strain DSR.

similar experiment to that of Fig. C.2 was performed, but this time the last centrifugation for the extract coming from Strain DSR was done at 9,000 rpm. The results, depicted in Fig. C.3, were very similar to the ones in Fig. C.2, therefore reaffirming that the presence of activity was in the particulate fraction of the extract. Cultures grown with the soluble fraction (supernatant after centrifugation) did not dechlorinate PCE. The particulate fraction of the extract is, therefore, necessary in the absence of extract from butyrate/PCE cultures but not sufficient to equal its activity.

To determine if the partial-response obtained with extract from Strain DSR was due to low levels of extract activity, a more concentrated extract was produced. This new extract was concentrated to 100x (the extract used in the experiments described above was 50x). The possible differences produced by the nutrients (YE and/or CA) with which Strain DSR was grown were also examined. As shown in Fig. C.4, the best response was obtained with cultures of Strain DSR grown with YE only, even though the cultures with YE and CA had higher cell density (data not shown). The poor response obtained with particulate fractions of extracts from cultures grown with both YE and CA has no explanation at this point. The sole presence of extract from Strain DSR, although it sustained dechlorination, was not completely satisfactory and it still indicated a limitation in the ability of the culture to dechlorinate and grow axenically without the extract from the butyrate/PCE culture. *"D. ethenogenes"* strain 195, grown in the presence of extract from Strain DSR only, was transferred twice under the same conditions (data not

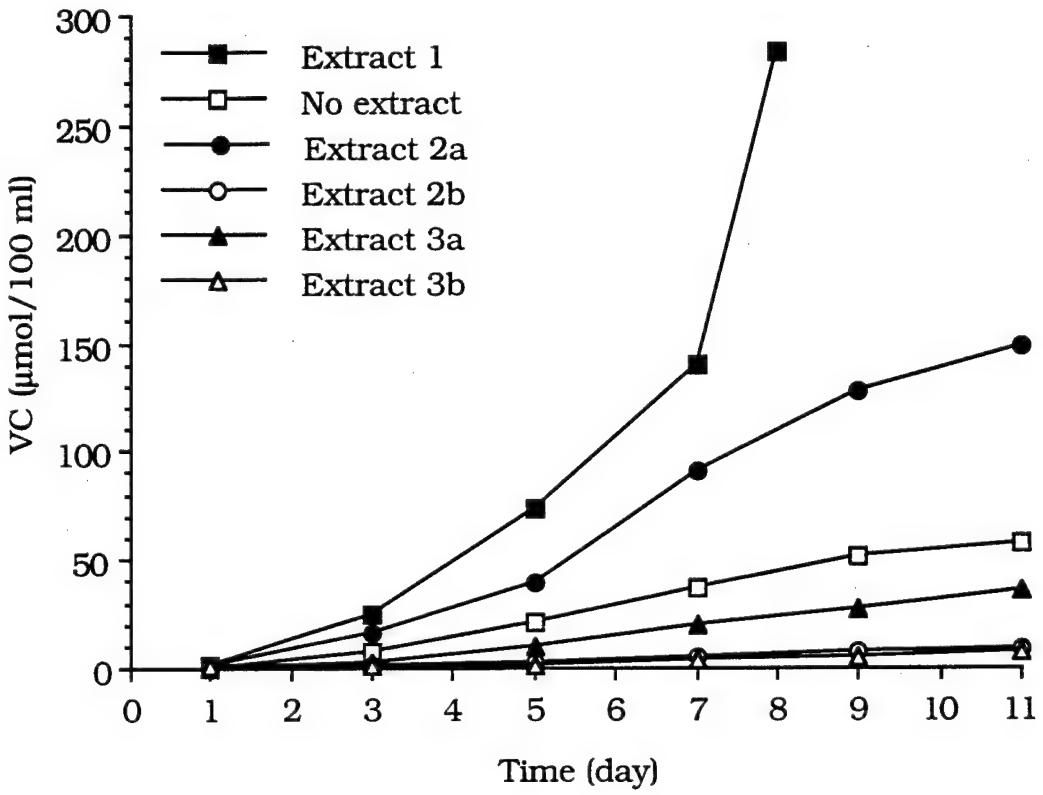

FIGURE C.4 Products from PCE dechlorination by *"D. ethenogenes"* strain 195 when grown axenically with different cell extracts. Extract 1 = from butyrate/PCE culture; extract 2a = particulate fraction of Strain DSR grown with YE (5 g/l); extract 2b = supernatant fraction of Strain DSR grown with YE (5 g/l); extract 3a = particulate fraction of Strain DSR grown with YE and CA (5 g/l each); extract 3b = supernatant fraction of Strain DSR grown with YE and CA (5 g/l each).

shown). Both consecutive transfers grew and dechlorinated PCE to VC and ETH, in manner similar to the culture used as inoculum, shown in Fig. C.4.

"D. ethenogenes" was grown in co-culture with Strain DSR, to corroborate their synthropism and to determine their dechlorinating performance together. As shown in Fig. C. 5, a first-generation culture transferred from an inoculum grown in medium containing extract from the butyrate/PCE culture showed good dechlorination if supplemented with the same extract, or with a small amount of yeast extract (0.2 g/l) and a pure culture of Strain DSR (2% v/v inoculum). The performance with Strain DSR was as good as that of the culture containing the extract. Cultures receiving neither extract nor Strain DSR cells produced minimal amounts of dechlorination products (data not shown).

"D. ethenogenes" strain 195 often requires two transfers into new medium before nutrient deficiencies become apparent, and therefore we transferred the co-culture a second time (data not shown). Cultures receiving no YE, so that no growth of the rod could occur, did not dechlorinate PCE to any significant extent. Control cultures receiving extract from the butyrate/PCE culture and ampicillin to inhibit growth of the rod showed, as usual, good dechlorination. Cultures amended with YE to allow growth of Strain DSR dechlorinated PCE at a lower rate than the cultures receiving extract from the butyrate/PCE culture, and eventually showed limitation, as evidenced by accumulation of TCE and DCEs. These results are in accordance with the ones found in Fig. C.5.

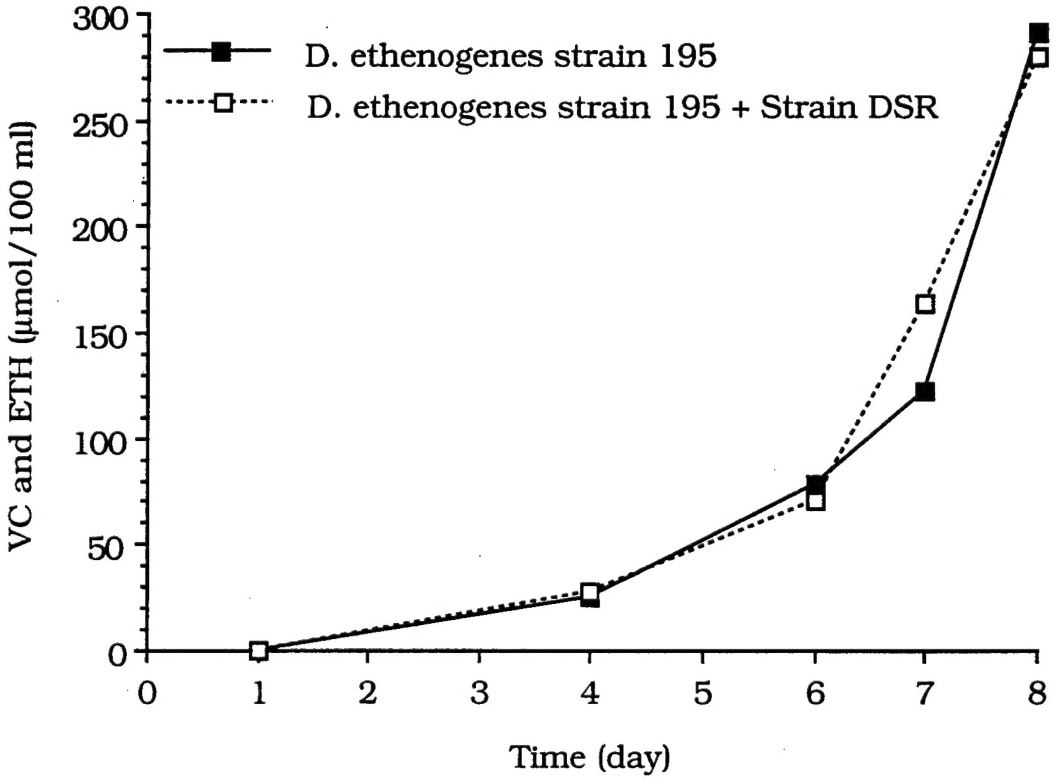

FIGURE C.5 PCE dechlorination products from *"D. ethenogenes"* strain 195 cultures to which additions of either extract from the butyrate/PCE culture or a culture of Strain DSR (plus 0.2 g/l YE) were made. All cultures were amended with 2 mM acetate, vitamins, and 25% v/v SS. The inoculum was a pure culture amended with butyrate pellet.

A further transfer of the co-culture to a third generation showed little dechlorination twelve days after inoculation (Fig. C.6), while control cultures receiving extract from the butyrate/PCE culture showed significant dechlorination (demonstrating that the culture was active under the right nutritional conditions). At Day 12, an addition of CA was made to the co-culture, which greatly increased growth of Strain DSR by Day 14, as observed by phase contrast microscopy. Several days later, the culture began rapid and extensive PCE dechlorination.

These results show that YE is required by Strain DSR in order to grow, because after adding CA to cultures of Strain DSR, only cultures containing YE showed increased turbidity and very significant rod growth. In cultures lacking YE, Strain DSR did not grow even after CA had been added, as observed by phase contrast microscopy. After CA was added to the co-culture, good PCE dechlorination was not observed until 11 days after the CA amendment. This probably means that only after Strain DSR entered advanced stationary (or death) phase did dechlorination by *"D. ethenogenes"* strain 195 start (the growth of a Strain DSR culture normally stops after about 60 hours). Either a product of CA metabolism or/and a molecule/s from the rod is/are probably required by the dechlorinator.

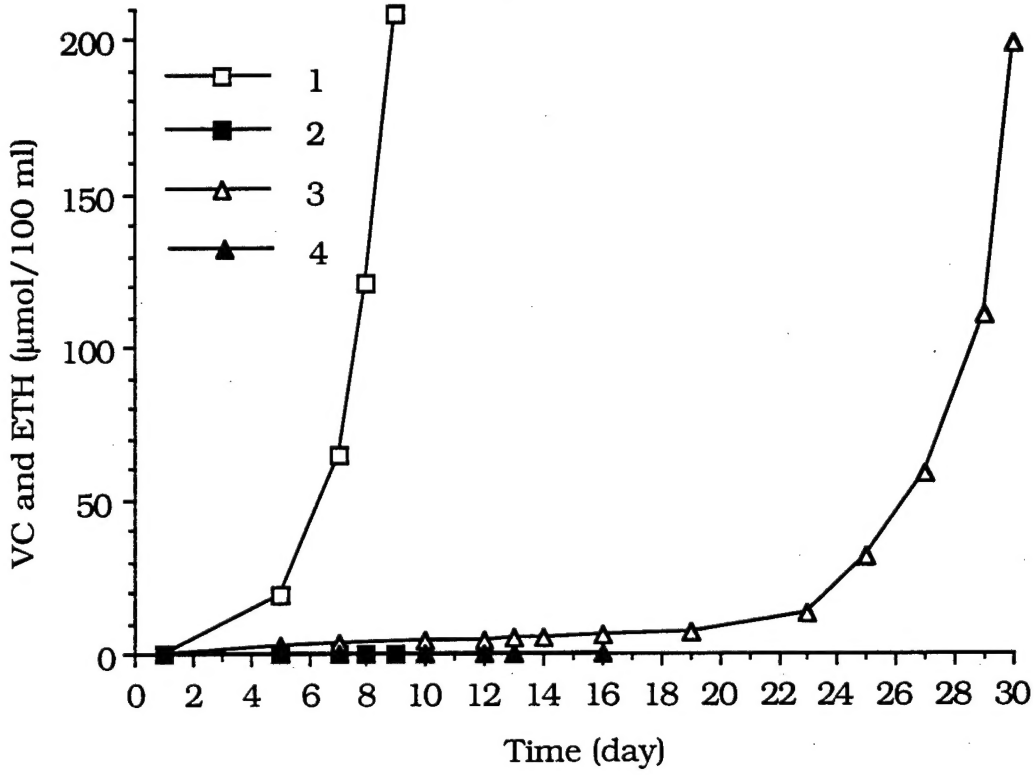

FIGURE C.6 PCE dechlorination products in third generation co-cultures. On Day 12, 0.2 g/l Casamino acids (CA), a substrate for the Strain DSR, was added to further stimulate its growth. 1 = *"D. ethenogenes"* strain 195 (in pure culture), with extract from the butyrate/PCE culture; 2 = *"D. ethenogenes"* strain 195 (in pure culture), with no cell extract; 3 = co-culture with 0.4 g/l YE; 4 = co-culture with no YE.

"Yes, there is a Nirvana;
it is in leading your sheep to a green pasture,
and in putting your child to sleep,
and in writing the last line of your poem"

Kahlil Gibran, Sand and Foam, 1970, p. 69
Alfred·A·Knoft, ed., New York